Drosophila embryos as a Model System to Study Bacterial Infection In Vivo

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Drosophila embryos as a Model System to Study Bacterial Infection *In Vivo*

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To My Family

"There is a single light of science, and to brighten it anywhere is to brighten it everywhere."

-Isaac Asimov

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Abstract

The fruit fly *Drosophila melanogaster* is recognized as the most widely established genetic model of immunity of the contemporary scientific era, exhibiting a high degree of conservation between *Drosophila* and mammalian innate immunity genes. However, whilst the majority of *Drosophila* immunity studies have previously been performed in adults and larvae, the embryo has recently emerged as a potentially viable model system; aiding *in vivo* studies and providing a more amenable system to undertake live imaging, hence evading many of the caveats associated with current immunity models. This project aimed to further develop the *Drosophila* embryo as a more potent and insightful immunity model, focusing on the immune response to bacterial infection.

Initial results demonstrated that the Stage 15 *Drosophila* embryo was able to mount a relatively robust immune response to bacterial infection. This included induction of antimicrobial peptide (AMP) genes upon a range of bacterial stimuli; a response which was able to effectively discriminate between differential types of bacterial infection via the characterized *Drosophila* systemic immunity pathways. Live-imaging studies also showed that the cellular immune response was functional within the Stage 15 embryo. Subsequently, immune competence was shown to arise at approximately mid-embryogenesis, under the control of 20-hydroxyecdysone (20-HE) signaling, as demonstrated by the partial rescue of AMP expression and bacterial clearance in early stage embryos upon 20-HE co-administration with infective agents. Further analysis of the global transcriptional response of the *Drosophila* embryo to infection and damage via microarray studies confirmed the immune potential of this system, but also permitted the identification of genes upregulated uniquely upon Gram-positive or Gram-negative infection. Moreover, wounding via sterile laser ablation induced significant upregulation of a subset of AMP genes and the a network of cuticular genes, providing an insight into the embryonic damage response. Parallel analysis of the hemocyte transcriptional profile upon infection and damage elucidated that these immune cells may play a role regulation of the immune response via 20-HE signaling and production of ROS, although this remains subject to further validation. As such, transcriptional profile analysis of the embryo has been successful in identifying candidate genes for further validation and study.

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Common Abbreviations

°C	Degrees Celsius
x g	x Gravitational Force
20-HE	20-Hydroxyecdysone
Aats-thr	Threonyl-tRNA Syntetase
AMP	Antimicrobial Peptide
ATP	Adenosine Triphosphate
Att	Attacin
BLASTn	Nucleotide Basic Local Alignment Search Tool
Bp	Base Pair
Bsh	Brain-specific homeobox
Btl	Breathless
Caps	Capricious
Cec	Cecropin
CO_2	Carbon Dioxide
Crq	Croquemort
C_t	Threshold Cycle
CYP	Cytochrome P450 Monoxygenase
DAP	Meso-Diaminopimelic Acid
Daw	Dawdle
Def	Defensin
DEPC	Diethyl Pyrocarbonate
dFADD	Drosophila Fas Associated Death Domain
DIAP2	Drosophila Inhibitor of Apoptosis 2
Dif	Dorsal-related Immunity Factor
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
Dpp	Decapentaplegic
Dpt	Diptericin
Drc	Drosocin
Dro	Drosomycin-like
Drs	Drosomycin
Duox	Dual Oxidase
Ecc15	*Erwinia carotovora carotovora 15*
FACS	Fluorescence Assisted Cell Sorting
FBS	Foetal Bovine Serum
FDR	False Discovery Rate
FOXO	Forkhead Box, Sub-group O
GADD45	Growth Arrest and DNA Damage-Inducible 45
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
GNBP	Gram-Negative Binding Protein
GO	Gene Ontology
GOrilla	Gene Ontology Enrichment Analysis and Visualisation Tool
Gprk2	G Protein-coupled Receptor Kinase 2
GTPase	Guanosine Triphosphatase
Hop	Hopscotch
hPa	Hectopascal

IKK	Inhibitor of κB Kinases
IM	Immune Inducible Molecule
IMD	Immune Deficiency
IRC	Immune Responsive Catalase
JAK	Janus Kinase
JH	Juvenile Hormone
JHa	Juvenile Hormone analogue
JNK	Jun-N-Terminal Kinase
lacZ	β-galactosidase
LB	Luria Bertani
LPS	Lipopolysaccharide
lquant	Loess and quantile normalisation
Lys	Lysine
MAPK	Mitogen-activated protein kinase
mas	Masquerade
mg	Milligrams
mbn-2	Malignant Blood Neoplasm 2
Milt	Milton
ModSP	Modular Serine Protease
MRSA	Methicillin-Resistant *Staphylococcus aureus*
Mtk	Metchnikowin
NCBI	National Centre for Biotechnology Information
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NHS	National Health Service
Niki	NimA-like Kinase
NPC	Niermann-Pick disease C
OBP	Odorant Binding Protein
OD_{600}	Optical Density at 600nm
OR	Odorant Receptor
PAMP	Pattern-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
P_c	Compensation Pressure
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGN	Peptidoglycan
PGRP	Peptidoglycan Recognition Protein
P_i	Injection Pressure
PO	Phenoloxidase
PPO	Prophenoloxidase
PPO-AE	Prophenoloxidase Activating Enzyme
Psh	Persephone
qPCR	Quantitative Polymerase Chain Reaction
Rel	Relish
Rfx	Regulatory Factor X
Rim	Rab3 Interacting Molecule
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
RT-qPCR	Reverse Transcription-Quantitative Polymerase chain reaction

S2	Schneider 2
SMART	Switching Mechanism at 5' End of RNA Templates of Reverse Transriptase
Snoo	Sno Oncogene
snRNA	Small nucleolar RNA
snz	Snazarus
Sp7	Serine Protease 7
Spz	Spatzle
Srp	Serpent
STAT92E	Signal Transducer and Activator of Transcription 92E
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
Tab	TAK1-Associated Binding Protein
TAK1	Tumour Growth Factor Activated Kinase 1
Trch	Trachealess
TEP	Thioester-Containing Protein
TGF-β	Transforming Growth Factor-β
T_m	Annealing Temperature
Tot	Turandot
Twdl	Tweedle
Upd	Unpaired
Vsn	Variance Stabilization Normalisation
Vvl	Ventral Veins Lacking
WHO	World Health Organisation

Chapter 1 – Introduction

1.1 *Drosophila* as a Model of Immunity

Drosophila has long been considered as an effective and potent model for studying and deciphering immune mechanisms (Lemaitre and Hoffmann, 2007). One of the earliest indications of *Drosophila* host defense against pathogens was upon the discovery of antibacterial factors in the fly hemolymph by Boman *et al*., (1972), demonstrating the existence of some inducible defense upon infection and giving the first insight that *Drosophila* could be applied more widely as a model of immunity. However, the wide application of *Drosophila* as an immune model can potentially be attributed to the high degree of conservation between the immune genes of the fly and those of mammalian systems (Hoffmann, 2003); for instance, the observation that Toll signaling components were able to regulate *Drosophila* innate immunity (Lemaitre *et al.,* 1996) facilitated the discovery of the existence of Toll-like receptors (TLRs) in mammalian systems that performed a homologous role (Medzhitov *et al.,* 1997). The recognition of the importance of this discovery is highlighted by this work being awarded the Nobel Prize in Physiology or Medicine in 2011 (Nobel Media AB, 2013). Thus by investigating the immune response in *Drosophila*, it is possible gain novel insight into the more complex mammalian immune response.

Thus far, knowledge of the *Drosophila* immune response has been procured via analysis of the host response after injection of pathogen or septic injury in larvae or adult flies (Tzou *et al.,* 2002). This response has been demonstrated to be complex, with oligonucleotide microarray studies revealing that several hundred genes are modulated in adult *Drosophila* after septic injury and fungal infection (De Gregorio *et al.,* 2001; Irving *et al.,* 2001; De Gregorio *et al.,* 2002). Moreover, the *Drosophila* system has the advantage of being genetically tractable, as well as allowing the possibility to infect large numbers of animals in a short time with highly reproducible results (Tzou *et al.,* 2002). Aside from *in vivo* infection studies, *in vitro* screens utilizing the *Drosophila*

Schneider 2 (S2) cell line have also become a crucial cornerstone of fly immunity studies. The primary cultures that form this cell line were isolated by Schneider (1972), from embryos between 20-24h old. S2 cells were shown to grow in a loose monolayer and be “macrophage-like” in appearance (Schneider, 1972). S2 cells subsequently found application to define and characterise pattern recognition receptors that are present on hemocytes, the *Drosophila* equivalent of mammalian macrophage (Ramet *et al.,* 2001; Ramet *et al.,* 2002), as well as encompassing an effective system to investigate the infection mechanisms employed by a variety of human bacterial pathogens (Cheng and Portnoy, 2003; Cheng *et al.,* 2005; Derre *et al.,* 2007). Another *Drosophila* cell line commonly used in immunity studies is the Malignant Blood Neoplasm 2 (mbn-2) line. This tumourous hemocyte line was originally derived from larvae with the mutation *lethal (2) malignant blood neoplasm*, which exhibit excessive hemocyte proliferation (Samakovlis *et al.,* 1992).

Subsequently, *Drosophila* immune models have been employed in a wide range of studies involving both Gram-positive and Gram-negative bacterial species. For instance, Blow *et al.* (2005) elucidated that infection of *Drosophila* with *Vibrio cholerae*, a Gram-negative bacteria and the causative agent of human cholera, very closely modeled the infection observed in humans, exhibiting many of the key traits of the disease. The study did not only provide confirmation of the cholera toxin as the key virulence factor, but also the use of *Drosophila* permitted the demonstration of the mechanism of action of the cholera toxin in a whole, live organism for the first time (Blow *et al.,* 2005), hence highlighting the potential use of *Drosophila* as an accurate and inexpensive model to study host susceptibility. Moreover, Needham *et al.*, (2004) used female adult *Drosophila* as a model host to aid the identification of two attenuated strains of *Staphylococcus aureus*, *pheP* and *perR*, and their subsequent infective potential. The study elucidated that not only that *S. aureus* was able to proliferate systemically within the fly, resulting in fly death (Needham *et al.,* 2004), but also that the flies could be rescued via antibiotic treatment. Many other Gram-positive bacterial species were also shown to kill *Drosophila* within this model, including *Streptococcus pneumoniae, Listeria monocytogenes* and *Streptococcus pyogenes* (Needham *et al.,* 2004), which

are causative agents of meningitis, food poisoning and scarlet fever respectively in humans (Rang *et al.,* 2003). *Drosophila* adults have also been employed to uncover further mechanistic details of immune signaling pathways (Rutschman *et al.,* 2000; Michel *et al.,* 2001; Rutschman *et al.,* 2001); for instance, screens of mutagenized *Drosophila* adult males performed by Ligoxygakis *et al.* (2002) resulted in the identification of Persephone, the first serine protease that was demonstrated to act upstream of the core Toll signaling pathway. Furthermore, S2 cells have been used to study the effects of the *L. monocytogenes* virulence protein listeriolysin O on intercytosolic growth and cell-to-cell spread (Cheng and Portnoy, 2003), as well as to conduct a genome wide RNA interference (RNAi) screen to identify host factors required for *L. monocytogenes* intracellular pathogenesis (Agaisse *et al.,* 2005) This further demonstrated the power of *Drosophila* as a high-throughput model system for studying bacterial infection.

Conversely, the infection need not be solely bacterial. Schneider and Shahabuddin (2000) demonstrated that a close relative of the malaria parasite, the protozoan *Plasmodium gallinaceum*, could effectively develop in *Drosophila*, with the formation of infectious sporozoites that demonstrate highly similar kinetics seen in other host species, such as the mosquito *Aedes aegypti,* thus substantiating results. Moreover, it was argued that the *Drosophila* system could be used for defining the genetic pathways required for both vector competence and the parasite sexual cycle (Schenider and Shahbuddin, 2000). Additionally, previous studies have used *Drosophila* as a high-throughput model to assess antifungal drug efficacy against *Aspergillus* virulence (Lionakis *et al.*, 2005) and *Drosophila* S2 culture cells have been used to gain greater insight into alphanodaviral RNA replication and the resulting upregulation of glycerophospholipid metabolism genes via transcriptomic studies (Castorena *et al.*, 2010). Therefore, the power of *Drosophila* as an immunity model is clear, enabling studies of a diverse range of pathogen types. Moreover, its wide application has facilitated the in-depth characterization of much of the *Drosophila* immune system itself, in turn providing novel insight into the complex mammalian immune response.

1.2 The *Drosophila* Immune System

1.21 Overview of the *Drosophila* Immune System

The *Drosophila* immune system itself consists solely of innate immune defense mechanisms, starkly lacking an equivalent of the adaptive immune reactions observed in vertebrate species (Hoffmann, 2003) and can be broadly sub-divided into two branches: the systemic and the cellular immune responses, as displayed in Figure 1.1. The systemic, or humoral, response is mediated within the fat body of the fly, the equivalent of the mammalian liver (Uvell and Engstrom, 2007), and refers to the challenge-induced synthesis and secretion of antimicrobial peptides (AMPs) (Lemaitre and Hoffmann, 2007). Two distinct pathways within the fat body recognize bacterial factors circulating in the hemolymph, such as peptidoglycan (PGN) (Gendrin *et al.,* 2009): these are the Toll and Immune Deficiency (IMD) pathways (Lemaitre and Hoffmann, 2007). Via these pathways, transcription factors, such as orthologs of Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells (NFκB), are activated to induce the expression of AMPs, which are subsequently secreted into the hemolymph to kill invading bacteria (Lemaitre and Hoffmann, 2007).

In contrast, the cellular response is mediated predominantly by hemocytes. The majority of these cells, more specifically termed plasmatocytes, are the *Drosophila* equivalent of mammalian macrophages (Kadandale *et al.,* 2010). These cells are actin-rich, and highly motile (Wood and Jacinto, 2007) and are crucial in mediating phagocytosis (Williams, 2007). Hemocytes also play roles in encapsulation, opsonisation and coagulation to further contribute to the cellular branch of the *Drosophila* immune response (Williams, 2007). Moreover, there is some evidence that hemocytes can interact with the systemic branch of the immune response, to further synergise and promote the production of AMPs (Agaisse *et al.,* 2003; Brennan *et al.,* 2007; Charroux and Royet, 2009; Shia *et al.,* 2009). Whilst the characterization of the systemic response has been extensive, and much is known regarding the mechanisms of its regulation and activation, relatively little is known about how the cellular

response is mediated and the precise role of the hemocyte during this process.

In addition to these two major immune branches, it is also necessary to consider the individual immune response mediated by epithelial tissue and the local immune response. Tissues that are in constant contact with the external environment have been demonstrated to locally express AMPs (Ferrandon *et al.,* 1998; Tzou *et al.,* 2000), as well as reactive oxygen species (ROS), which are directly toxic to microbes and hence render pathogens inactive (Cerenius and Söderhäll, 2004; Kounatidis and Ligoxygakis, 2012).

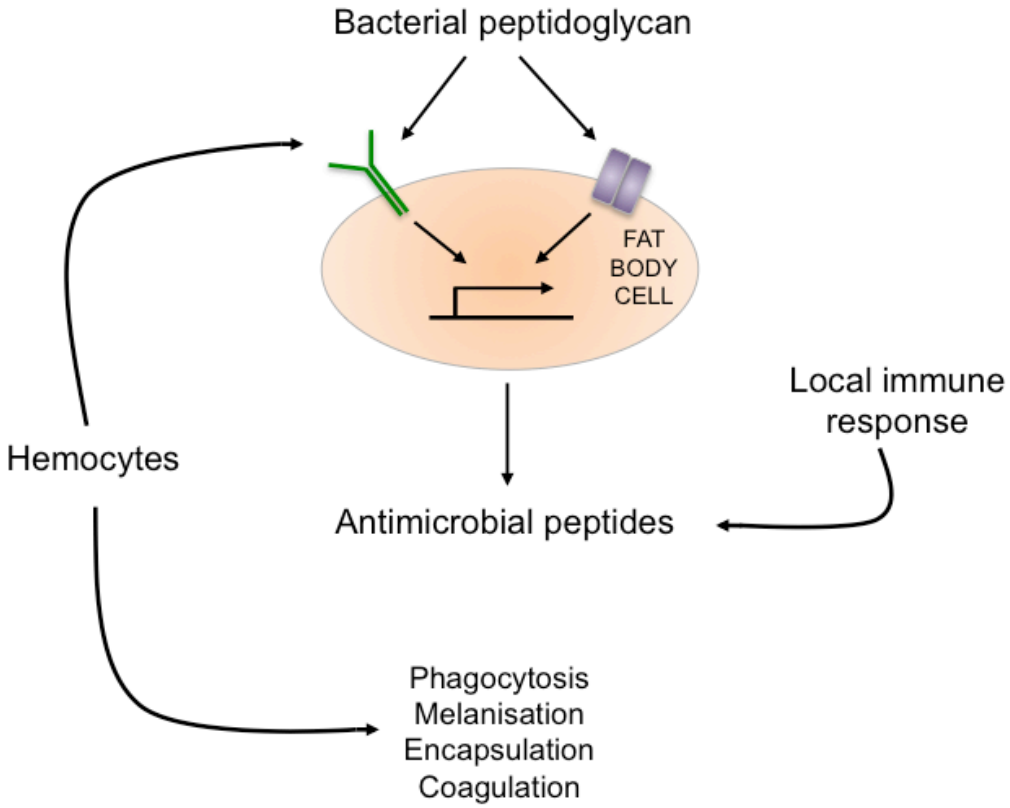

Figure 1.1: An Overview of the *Drosophila* Immune System. The immune response in *Drosophila* consists of two major branches: the systemic and cellular responses, mediated by the fat body and hemocytes, the *Drosophila* equivalent of the mammalian liver and macrophages, respectively. The systemic response is activated when circulating bacterial factors are recognized by the Toll and Immune deficiency (IMD) pathways; these activate NFκB analogues to induce expression of anti-microbial peptides (AMPs). Hemocytes can feedback to the fat body to promote the systemic response, and are also implicated in other distinct immune processes. There is also a peripheral local response, where AMP expression is localized to specific tissues.

Therefore, the *Drosophila* immune response is multi-faceted, displaying a range of host mechanisms to successfully eliminate invading pathogens which are analogous to many of the innate immunity mechanisms observed in mammalian systems, such as the clearance of bacteria by professional phagocytes and the production of AMPs (Yang *et al.,* 2001; Selsted and Ouellette, 2005).

1.2.2 Antimicrobial Peptides

The most prominent protein effectors of the *Drosophila* immune response are the AMPs. AMPs are small cationic proteins, with a diverse range of activities against bacteria and fungi (Imler and Bulet, 2005). The definition of an AMP itself includes all peptides that can kill microbes, but does not encompass enzymes that neutralize microbes via hydrolytic activity (Maroti *et al.,* 2011). Of the range of AMPs currently characterised, some exhibit a narrow spectrum of activity, whilst others are able to react to a large range of Gram-negative and Gram-positive bacteria, as well as fungi, viruses and parasites (Maroti *et al.,* 2011). Some AMPs, such as Drosomycin, are chemically highly stable due to the presence of intramolecular disulphide bridges within their secondary structure and can be detected in the hemolymph for several weeks post-challenge (Uttenweiler-Joseph *et al.,* 1998). It has been suggested by Wei *et al.,* (2009) that precise regulation of transient AMP expression can be attributed to the stability of individual AMP mRNA transcripts, creating a dynamic and balanced system of systemic AMP production. AMPs with a broader antimicrobial spectrum were shown to have transcripts that decayed more quickly (Wei *et al.,* 2009). However, compared to other components of the immune response, AMPs as a whole are deployed relatively rapidly (Shai, 2002). This is crucial to the host survival, as other branches of the immune response may occur relatively slowly in comparison to the kinetics of microbial proliferation and as such would allow the infection to grow un-checked (Shai, 2002). Moreover, the fact that AMP genes are conserved throughout the animal and plant kingdoms would suggest that AMPs played a crucial and

fundamental role in the evolution of complex multicellular organisms (Zasloff, 2002).

The precise mechanism of AMP damage and microbial killing is still somewhat controversial. Their microbicidal mechanism of action has been extensively characterised *in vitro* (Brogden, 2005), but any comprehensive picture of how these proteins operate *in vivo* has remained elusive (Junell *et al.,* 2010). What is clear is that peptide-mediating killing can be rapid; in fact some linear α-helical antimicrobial peptides kill bacteria so rapidly that it can make characterization of the steps preceding cell death very difficult (Boman, 1995). Several hypotheses have suggested that transmembrane pore formation may hold the key to AMP action (Brogden, 2005). Within this process, AMPs must be attracted to bacterial surfaces, probably due to electrostatic attractive forces between the positively charged AMP and negatively charged outer leaflet of the bacterial lipid bilayer (Zaslof, 2002). Once sufficiently close to the bacterial surface, AMPs must traverse the capsular polysaccharide landscape before they can effectively interact with the outer membrane (Brogden, 2005). They are consequently free to interact with the lipid bilayers, and many models exist which postulate how this may occur. The central elements of these models include binding of AMPs to the bacterial membrane, insertion of AMPs into the bilayer and the subsequent formation of a transmembrane pore, leading to bacterial cell lysis and elimination (Brogden, 2005). Nevertheless, the actual mechanism of pore formation appears to differ depending on the genre of AMP; some AMPs have been shown to form a ‘barrel’ type pore composed of α helices around a central pore (Yang *et al.,* 2001), whereas others induce a ‘toroidal’ pore whereby peptide inserts into the membrane and causes continual bending of the lipid monolayers (Matsuzaki *et al.,* 1996). Moreover, an alternative model suggests that AMPs may also disrupt bacterial membranes in a detergent-like manner (Almedia and Pokorny, 2009), whereby ‘carpet’ coverage of the target cell in AMP molecules leads to membrane disintegration (Shai, 1999; Ladokhin and White, 2001). There is also increasing evidence that AMPs may also have intracellular targets. For example, Drosocin has been demonstrated to bind to DnaK, a heat shock

protein (Otvos *et al.,* 2000), as well as preventing the refolding of misfolded proteins (Kragol *et al.,* 2001). Other intracellular AMP action could potentially include the inhibition of bacterial cell wall synthesis (Brotz *et al.,* 1998), alteration of the cytoplasmic membrane and inhibition of septum formation (Salomon *et al.,* 1992; Shi *et al.,* 1996; Subbalakshmi *et al.,* 1998), inhibition of DNA, RNA or protein synthesis (Lehrer *et al.,* 1989; Yonezawa *et al.,* 1992; Boman et al., 1993; Park *et al.,* 1998; Subbalakshmi *et al.,* 1998; Patrzykat *et al.,* 2002) and activation of autolysin and phospholipases (Bierbaum and Sahl, 1987; Zhao and Kinnunen, 2003).

Table 1.1: *Drosophila* Antimicrobial Peptides and their Effectiveness against Specific Pathogen Categories

Antimicrobial peptide (AMP) class	Number of genes	Predominant pathogen effectiveness	Estimated hemolymph concentration (μM)(3.s.f.)
Attacins	4	Gram-negative bacteria	ND
Cecropins	4	Gram-negative bacteria	20.0
Defensin	1	Gram-positive bacteria	1.00
Diptericins	2	Gram-negative bacteria	0.50
Drosocin	1	Gram negative bacteria	40.0
Drosomycins	7	Fungi Gram-positive bacteria	100
Metchnikowin	1	Fungi	10.0

A range of antimicrobial peptides (AMPs) is expressed by *Drosophila* in response to Gram-negative and Gram-positive, as well as fungal pathogens, with hemolymph concentrations varying in the micromolar concentration range post infection (ND, not determined) (Lemaitre and Hoffmann, 2007).

There are currently 20 characterised immune-inducible AMPs in *Drosophila*, grouped into 7 classes, as depicted in Table 1.1 (Maroti *et al.,* 2011). The *Drosophila* antimicrobial response is not aspecific; it is able to distinguish between various classes of microorganisms (Lemaitre *et al.,* 1997). Thus,

dependent on the nature of the pathogen, specific AMPs will be induced to effectively aid the clearance of the infection. In *Drosophila*, the AMPs can generally be sub-divided into those responsive to Gram-negative infections and those stimulated by Gram-positive and fungal species (Lemaitre *et al.,* 1997). Yet, it remains a major challenge to understand the relative contributions of individual AMPs towards the total host defense and the potential synergy between different individual AMPs (Lemaitre and Hoffmann, 2007).

A. Gram-negative AMPs

Of the 20 different AMPs identified within the *Drosophila* genome, those that are induced in response to infection by Gram-negative bacterial pathogens comprise Attacins, Cecropins, Diptericins and Drosocin. Whilst there is only one copy of the *Drosocin* (*Drc*) gene contained within the *Drosophila* genome (Bulet *et al.,* 1993; Charlet *et al.,* 1996), the others are encoded by multi-gene families (Deng *et al.,* 2009).

Attacins are one of the most taxonomically widespread AMP classes (Ando *et al.,* 1987; Sugiyama *et al.,* 1995; Lazzaro and Clark, 2001). *Att* genes were first identified in the genome of *Drosophila* by Åsling *et al.,* 1995, who were able to isolate a gene sequence from *Drosophila* which showed sequence homology to *AttA* genes from other insect species. Subsequently, they were able to localize two *Att* genes to the second *Drosophila* chromosome, which were later termed *AttA* and *AttB* (Åsling *et al.,* 1995). Basic Local Alignment Search Tool (BLAST) studies also permitted the discovery of two further isoforms, termed *AttC* and *AttD* (Hedengren *et al.,* 2000; Lazzaro and Clark, 2001). In terms of structure, Att proteins are Glycine-rich (Hedengren *et al.,* 2000) and approximately 190 amino acids in length (Lazzaro and Clark, 2001). In solution, they adopt a random coil structure (Gunne *et al.,* 1990). This feature may be attributed to the lack disulphide bonds in the secondary structure of Att, and the resulting lack of conformational constraint may in turn allow relatively free amino acid substitution (Lazzaro and Clark, 2001). Regarding gene structure, *AttA* and *AttB* exhibit 96% sequence identity, and

Lazzaro and Clark (2001) reported that they were arranged in a head-to-tail fashion, separated by 1.1kb. On the other hand, *AttC* is located further upstream and only shows 67-73% sequence identity to *AttA* (Hedengren *et al.,* 2000; Lazzaro and Clark, 2001). Consequently, Lazzaro and Clark (2001) postulated that *AttC* may be differently targeted or processed compared to *AttA* and *AttB*, although the relatively high degree of sequence similarity may infer some common function for all three isoforms. Even further removed is *AttD*, which only demonstrates 33% sequence identity to *AttA* (Lazzaro and Clark, 2001) and is located on the third *Drosophila* chromosome (Hedengren *et al.,* 2000). Moreover, *AttD* appears to exhibit a truncation at the 5' end of the gene, in comparison to the other *Att* isoforms (Hedengren *et al.,* 2000). Thus it lacks the N-terminal signal and the propeptide sequences that are required for activation (Hedengren *et al.,* 2000) and it is questionable whether the resulting protein is subsequently secreted and functional as an AMP. However, regardless of these differences in structure, there is little or no expression of any *Att* isoform in unchallenged flies; only upon immune challenge with Gram-negative bacterial species are they strongly induced, at approximately 6 hours post infection (Hedengren *et al.,* 2000).

Drosophila Diptericins are another group of AMPs that are glycine-rich and bear some sequence homology to Attacins, suggesting a common origin (Wicker *et al.,* 1990). Initially, only one Diptericin (*Dpt*) gene was identified within the *Drosophila* genome (Imler and Bulet, 2005). This gene produces an 83-residue product, with an O-glycosylation on a threonine residue within its N-terminal domain (Imler and Bulet, 2005). The expression of this gene was subsequently demonstrated to follow classical acute phase response kinetics upon *E. coli* infection (Wicker *et al.,* 1990), concordant with the discovery that specific motifs in the proximal *Dpt* promoter exhibited homology to the mammalian *cis*-regulatory sequences involved in expression of acute phase response genes (Georgel *et al.,* 1993). Moreover, *Dpt* was demonstrated to exhibit antibacterial activity against *E. coli*, and was shown to be upregulated in *Drosophila* adults and larvae from 2 hours post inoculation (Wicker *et al.,* 1990). More recently, a second novel *Dpt*-like gene was partially sequenced (Lee *et al.,* 2001; Imler and Bulet, 2005). This novel gene was determined to

be a paralog of *Dpt* (Lee *et al.,* 2001), and subsequently was designated *DptB* (Imler and Bulet, 2005). Similar to its paralog, *DptB* is rapidly induced upon infection with *E. coli* and secreted specifically from the fat body (Lee *et al.,* 2001). Moreover, its proximal promoter region exhibited features that are typically associated with the correct expression of antibacterial genes, such as κB binding sites (Lee *et al.,* 2001).

Another AMP class induced by Gram-negative microbes is the Cecropin multi-gene family. Cecropins are 29-42 amino acid residues in size; they are linear and cysteine-free AMPs (Imler and Bulet, 2005). The *Drosophila Cecropin* (*Cec*) locus was originally cloned by Kylsten *et al.* (1990), and it was consequently discovered that this region contained a dense cluster of three functional cecropin genes, termed *CecA1*, *A2* and *B*, and two pseudogenes, annotated *Cecφ1* and *Cecφ2* (Samakovlis *et al.,* 1990; Samakovlis *et al.,* 1992). All three of the functional genes were strongly induced in 3rd instar larvae, pupae and adults upon infection with *Enterobacter cloacae*, from typically undetectable basal levels (Samakovlis *et al.,* 1990). However, a relatively low level of *Cec* transcript was detected in early pupae and occasionally a low, variable expression in adults (Samakovlis *et al.,* 1990). *CecB* appeared to be less inducible in larvae and adults, and instead appeared to be preferentially induced in early pupae, due to induction of *CecB* by residing gut bacteria (Samakovlis *et al.,* 1990). As with the other Gram-negative AMPs, the primary site of production of the *Cec* family members was determined to be the fat body, with studies also identifying approximately 5-10% of the hemocyte population that express *Cec* transcript (Samakovlis *et al.,* 1990). Subsequent studies also elucidated the presence of a fourth *Cec* gene, approximately 3kb upstream of *CecB*. The subsequently termed *CecC* was then cloned and demonstrated to show a similar pattern of expression to *CecB*, both basally and upon induction with bacteria (Tryselius *et al.,* 1992). However, whilst expression upon infection is localized to the fat body, that during metamorphosis may be sited at the hindgut and more specific fat body foci (Tryselius *et al.,* 1992). It is also noteworthy that, whilst *Cec* genes are preferentially induced by Gram-negative microbes, such as *E. coli*, they are

also weakly induced by Gram-positive bacterial species and fungi (Lemaitre *et al.,* 1997; Ekengren and Hultmark, 1999).

On the other hand, Drosocin (Drc) does not exhibit the multi-gene family of the other Gram-negative responsive AMPs; as mentioned above, only one copy of the *Drc* gene has been characterised in the *Drosophila* genome to date (Bulet *et al.,* 1993). Drc is much smaller than other AMPs, consisting of only 19 amino acid residues (Charlet *et al.,* 1996). Amongst its other structural features, it has been shown to be proline-rich and carries an O-glycosylated substitution at Thr11 (Bulet *et al.,* 1996); a feature that it shares with *DptA* (Imler and Bulet, 2005). It is thought that this substitution may affect the conformation of Drc protein random coil turns in aqueous solution (McManus *et al.,* 1999) and thus may represent a post-translational modification, which is required for full antibacterial activity of the protein (Bulet *et al.,* 1993; Bulet *et al.,* 1996; Charlet *et al.,* 1996). Similar to the other Gram-negative AMP genes, *Drc* is not constitutively expressed in naïve, unchallenged larvae, pupae or adult flies (Charlet *et al.,* 1996). Upon infection by septic injury, rapid and intense *Drc* expression is observed in the fat body, with kinetics comparable to those of the *Dpt* genes (Charlet *et al.,* 1996). The mbn-2 cell line was also shown to express *Drc* after treatment with lipopolysaccharide, a component of Gram-negative bacterial cell walls (Charlet *et al.,* 1996), suggesting that hemocytes may also express *Drc* upon infection. However, this *in vitro* prediction was not concordant with *in vivo* infection studies using transgenic *Drc-lacZ* flies (Charlet *et al.,* 1996), thus the capacity for hemocytes to express *Drc* upon infection remains contentious.

B. Gram-positive and fungal AMPs

Conversely, upon infection with Gram-positive bacterial or fungal infection a set of AMPs, distinct from those induced by Gram-negative bacteria, is induced. This comprises the Drosomycins, Metchnikowin and Defensin. Whilst there only single copies of the *Metchnikowin* (*Mtk*) and *Defensin* (*Def*) genes within the *Drosophila* genome (Levashina *et al.,* 1995; Dimarcq *et al.,* 1994), the Drosomycin (Drs) gene is clustered with 6 additional genes that show highly similar sequence homology (Deng *et al.,* 2009). Whilst other forms of

Def have been reported to be present in many other insects, Drs and Mtk are specific to *Drosophila* (Imler and Bulet, 2005).

Drosomycin is a 44 amino acid residue protein, whose 4 intramolecular disulfide bridges form a cysteine-stabilised αβ motif (Michaut *et al.,* 1996; Landon *et al.,* 1997). This conforms a great degree of stability on the Drs protein, and hence it is detectable for up to three weeks post challenge in flies (Utterweiler-Joseph *et al.,* 1998). *Drs* is constitutively expressed in *Drosophila* adults and larvae (Lemaitre *et al.,* 1997; Levy *et al.,* 2004), but is further upregulated by immune challenge (Deng *et al.,* 2009). Some early studies argued that *Drs* was solely induced by filamentous fungal infection, and not by challenge with either Gram-positive bacteria or yeast (Fehlbaum *et al.,* 1994), but subsequent studies have discovered that induction of *Drs* is also possible via immune challenge with a variety of Gram-positive bacteria in *Drosophila* adults (Bischoff *et al.,* 2004). Moreover, *Drosophila* larvae challenged with Gram-negative bacteria were also able to weakly upregulate *Drs* (Lemaitre *et al.,* 1997). As mentioned above, *Drs* forms part of a multi-gene cluster on the 3L chromosome arm (Jiggins and Kim, 2005; Yang *et al.,* 2006). The other 6 members of this cluster are genes that display high sequence homology to the *Drs* gene, and hence have been termed *Dro1, Dro2, Dro3, Dro4, Dro5* and *Dro6* (Deng *et al.,* 2009). The predicted structures of these genes also contain cysteine residues which may promote the formation of disulphide bridges and hence lead to structure stabilization (Landon *et al.,* 1997). Expression of these genes in *E. coli* and purification of the resulting proteins lead to the isolation of peptides with antifungal activity, with the exception of that originating from *Dro6* (Yang *et al.,* 2006). However, despite their similarities in structure and activity, these *Drs*-like genes are not necessarily co-expressed during the individual phases of the *Drosophila* lifecycle. For example, whilst *Drs* ad *Dro2* were discovered to be expressed in larvae, pupae and adult flies, *Dro3*, *Dro4* and *Dro5* were shown only to be expressed in the larval and adult stages of the *Drosophila* lifecycle (Tian *et al.,* 2008). *Dro1* and *Dro6* expression was not detected in any stage of the *Drosophila* lifecycle (Tian *et al.,* 2008). Moreover, knowledge concerning the *Drs*-like genes is relatively limited; the expression

and regulation of each member in response to microbial challenge or damage has not been fully characterised (Tian *et al.,* 2008). Some studies have sought to rectify this deficit; Deng *et al.* (2009) investigated the expression patterns of Drs family members to injury and microbial infection in *Drosophila* adults. *Drs, Dro2, Dro3, Dro4* and *Dro5* showed constitutive expression (Deng *et al.,* 2009), consistent with previous work focusing solely on *Drs* (Levy *et al.,* 2004). *Dro2*, *Dro3* and *Dro5* were upregulated by simple injury, whereas *Drs* was discovered to be the only family member to be further induced upon microbial challenge (Deng *et al.,* 2009). In concordance with previous work (Tian *et al.*, 2008), *Dro1* and *Dro6* were not transcribed upon any microbial or injury treatments (Deng *et al.,* 2009). These results mirror the discovery of binding sites for NFκB-related elements within the promoter regions of *Drs, Dro2, Dro3* and *Dro5* (Deng *et al.,* 2009), although the precise mechanism responsible for this differential regulation upon infection and damage is yet to be determined.

In contrast to Drs, Def is selectively effective against Gram-positive bacterial species (Dimarcq *et al.,* 1994). Structurally, it is a 40-residue peptide, with three internal disulphide bridges (Imler and Bulet, 2005). Only one *Def* gene has been identified in *Drosophila* (Imler and Bulet, 2005) and studies have shown that *Def* expression post infection occurs predominantly in the fat body (Dimarcq *et al.,* 1994). It is possible that *Def* expression may also be sited within hemocytes upon microbial challenge, when considering that LPS was able to induce *Def* expression in the mbn-2 cell line (Dimarcq *et al.,* 1994). In terms of microbicial action, changes in physiological salt concentration may have an effect on Def activity in *Drosophila*, considering that other homologous insect Defensins, such as from the blowfly *Protophormia*, cause dramatic bacterial lysis at micromolar concentrations when physiological salt concentration is low (Imler and Bulet, 2005). It has been postulated that this may enable disruption of the permeability of bacterial membranes, resulting in a partial depolarization of the inner membrane, a decrease in cytoplasmic ATP and thus inhibition of respiration (Cociancich *et al.,* 1993).

The final class of Gram-positive AMP consists of Mtk; a proline-rich AMP (Levashina *et al.,* 1995), which is transcribed from a single gene (Imler and Bulet, 2005). In terms of other structural features, it is a linear 26 amino acid peptide that lacks cysteine residues (Imler and Bulet, 2005). Mtk in not detectable in unchallenged 3rd instar larvae and pupae, and detected at very low basal levels in unchallenged adult flies (Levashina *et al.,* 1995). However, the expression of *Mtk* is the subject of much divided opinion within the scientific literature. Some studies show that immune stimulation with LPS was sufficient to strongly drive transcription of *Mtk* at all stages of development investigated; after 3 hours in 3rd instar larvae, 8 hours in pupae and 6 hours in adult flies (Levashina *et al.,* 1995). Other literature has suggested that *Mtk* is strongly induced in flies with either Gram-negative bacteria, Gram-positive bacteria or fungal pathogens (Levashina *et al.,* 1995; Lemaitre *et al.,* 1997), with the highest levels of expression exhibited in flies infected with Gram-positive bacteria and fungal species (Lemaitre *et al.,* 1997). Furthermore, during its purification Mtk was reported to affect the growth of the filamentous fungi *Neurospora crassa*, as well as that of the Gram-positive bacterium *Micrococcus luteus*, but the resulting recombinant Mtk was found only to exhibit antifungal activity (Imler and Bulet, 2005). Therefore, Mtk has now been classed by many as simply an antifungal agent (Imler and Bulet, 2005). Regardless of actual immune stimulus, Levashina *et al.* (1998) used a transgenic *Drosophila Mtk-GFP* fusion line to demonstrate that expression of Mtk occurs within the fat body of 3rd instar larvae and adult flies after immune challenge, as well as in some populations of hemocytes in specific 3rd instar larvae. The protein itself has been noted for its stability and persistence within the *Drosophila* system; for instance, high levels are maintained for 2 to 3 days after infection with the entomopathogenic fungus *Beauveria bassiana* (Lemaitre *et al.,* 1997).

Accordingly, AMPs form a cornerstone of the *Drosophila* systemic immune response, representing a major mechanism by which the insect is able to effectively fight a wide range of pathogens, although the precise mechanism by which this is mediated requires some clarification. Moreover, understanding how production of these peptides is mediated via the systemic immunity

pathways has given novel insight into how innate immunity is regulated. This view is perpetually expanding, hence demonstrating the complexity of the innate immune response itself.

1.3 Systemic Immunity Signaling Pathways

1.3.1 The Immune Deficiency (IMD) Pathway

A. Pathway Overview

The IMD pathway was initially identified via a mutation named *immune deficiency* (Lemaitre and Hoffmann, 2007), which in turn severely impaired the expression of a variety of Gram-negative AMPs, and marginally impaired Drs induction. As a result, these flies often succumbed to Gram-negative bacterial infection, but were more resistant to fungi and Gram-positive bacteria, leading to the confirmation that the systemic IMD pathway activation was specific only to Gram-negative bacterial pathogens, such as *E. coli* (Lemaitre *et al.,* 1995). More recent studies have elucidated that circulating *meso*-diaminopimelic acid (DAP)-type peptidoglycan typical of Gram-negative bacteria is detected by peptidoglycan recognition proteins (PGRPs) (Kaneko *et al.,* 2004; Stenbak *et al.,* 2004). These receptors consequently recruit and signal via the IMD adaptor, which interacts with the *Drosophila* Fas-Associated Death Domain (dFADD). As a result, dFADD binds the caspase Dredd. The *Drosophila* IκB Kinase (IKK) complex phosphorylates Relish, which has been proposed to aid the cleavage of Relish by Dredd (Silverman *et al.,* 2000; Erturk-Hasdemir *et al.,* 2009). The resulting cleavage allows the subsequently released Relish to translocate to the nucleus and facilitate the transcription of Gram-negative AMP genes, such as Dpt and Drc. Moreover, the activation of the IKK complex itself is postulated to be activated in an IMD dependant fashion, facilitated by dIAP2, an Inhibitor of Apoptosis Protein, and dTAK1, a Mitogen-Activated Protein 3 Kinase, but the precise mechanisms that link these components are currently unknown (as reviewed in Lemaitre and Hoffmann, 2007). Furthermore, the molecular organization of the pathway is currently poorly understood due to a lack of information concerning the subcellular localization

of many pathway components, thus ensuring the difficulty in performance of epistatic analyses (as reviewed in Lemaitre and Hoffmann, 2007). A schematic of the IMD pathway is depicted in Figure 1.2.

B. Recognition

IMD pathway activation is achieved through the recognition of DAP-type PGN (Leulier *et al.,* 2003; Choe *et al.,* 2005), which is a component of most Gram-negative species' cell walls, although members of some Gram-positive genera such as *Listeria* and *Bacillus* also have cell walls comprised of this material and hence are able to activate IMD signaling (Lemaitre *et al.,* 1997; Stenbak *et al.,* 2004; Kleino and Silverman, 2013). This recognition and subsequent initiation of IMD signaling is mediated via the receptors PGRP-LC, a transmembrane receptor, and PGRP-LE, a circulating extracellular and intracellular receptor (Kaneko *et al.,* 2006), which act synergistically (Takehana *et al.,* 2004).

There are three alternative splice variants of PGRP-LC, which result in three proteins with identical transmembrane and cytoplasmic regions, but different PGRP domains (Werner *et al.,* 2003; Stenbak *et al.,* 2004): these are referred to as PGRP-LCx, LCy and LCa. PGRP-LCx and PGRPLCa are relatively well characterised and have been shown to display differential binding specificities for DAP-type PGN (Kleino and Silverman, 2013). Kleino and Silverman (2013) propose that the polyvalent nature of the DAP-type PGN ligand may cluster multiple PGRP-LCx receptors to facilitate downstream signaling events. PGRP-LCa is not able to bind DAP-type PGN in the same manner, due to the absence of a cleft of sufficient depth to accommodate the polymeric nature of PGN (Kleino and Silverman, 2013). Thus it has been shown that this variant is able to bind a monomeric fragment of DAP-type PGN, termed tracheal cytotoxin (TCT) via PGRP-LCx, forming a ligand-induced heterodimer (Chang *et al.,* 2004; Kaneko *et al.,* 2005; Mellroth *et al.,* 2005; Chang *et al.,* 2006). Conversely, the precise function of PGRP-LCy has not been fully determined, but recent research suggests that it may function as an antagonist of the IMD response (Werner *et al.,* 2003; Neyen *et al.,* 2012). In contrast, PGRP-LE is

able to recognize DAP-type PGN that enters the host cell cytoplasm, through an as of yet unidentified mechanism (Kaneko and Silverman, 2005; Yano and Kurata, 2011), or that originating from intracellular pathogens (Yano *et al.,* 2008). PGRP-LE is also able to cooperate with extracellular domains of PGRP-LC in the recognition of extracellular DAP-type PGN and TCT (Takehana *et al.,* 2002; Kaneko *et al.,* 2006; Lim *et al.,* 2006). Despite our understanding of how these receptors are able to bind their bacterial ligand is expanding, it is currently unclear how DAP-type PGN binding elicits downstream IMD signaling. However, there are suggestions that the N and C-termini regions of the PGRPs may play a role in regulating this process (Choe *et al.,* 2005; Maillet *et al.,* 2008) and that conformational change of receptors and multimerisation may be required to interact with downstream signaling adaptors (Kleino and Silverman, 2013).

In addition, other PGRP isoforms are also able to act as negative regulators of IMD signaling. Receptor isoforms, such as PGRP-LB, -SC1 and -SC2, have amidase activity (Mellroth *et al.,* 2005; Zaidman-Remy *et al.,* 2006) and are hence able to degrade DAP-type PGN to attenuate IMD pathway activation (Mellroth *et al.,* 2003; Bischoff *et al.,* 2006). PGRP-LB is initially expressed in fat body tissue and is subsequently secreted and circulates in the hemolymph (Zaidman-Remy *et al.,* 2006). Similarly, PGRP-SC1/2 are expressed in the gut epithelial tissue and released into the lumen (Kleino and Silverman, 2013). Moreover, PGRP-LF is also able to downregulate IMD signaling (Persson *et al.,* 2007; Maillet *et al.,* 2008; Basbous *et al.,* 2011), although precisely how this isoform facilitates such a response is uncertain. Some evidence would suggest that PGRP-LF acts as a decoy receptor (Persson *et al.,* 2007), whereas other data would suggest that this isoform is not able to bind PGN but binds PGRP-LCx to compete for a place within the its multimers, hence directly blocking signal transduction (Basbous *et al.,* 2011).

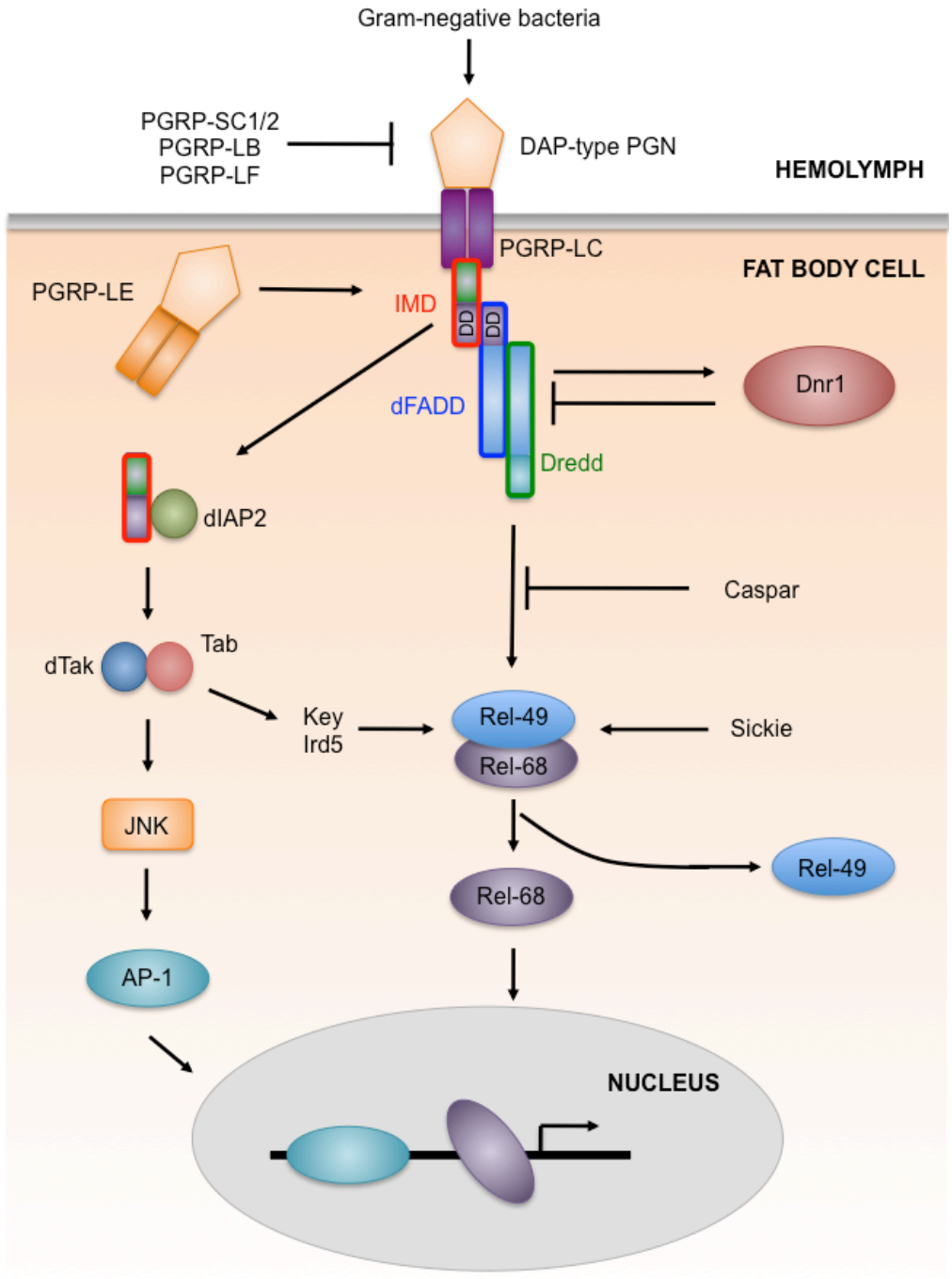

Figure 1.2: An Overview of the Immune Deficiency (IMD) Activation in the *Drosophila* fat body. PGRP-LC and -LE recognize Gram-negative bacterial factors circulating in the hemolymph, including DAP-type peptidoglycan (DAP-type PGN) and signal to the IMD adaptor, which via interaction with the death domains (DD) of dFADD activates the Dredd caspase. Dredd and Sickie interact with Relish (Rel) -49 and Rel-68 thus causing release of the Rel transcription factor. This is then able to translocate to the nucleus and induce the expression of Gram-negative specific AMPs. IMD may also become ubiquitinated by *Drosophila* Inhibitor of Apoptosis 2 (dIAP2), leading to the recruitment of the dTak/Tab2 complex and subsequently facilitating the activation of Relish by the IKK complex (Key/Ird5). Among the potential negative regulators of the pathway, PGRP isoforms may act as decoy receptors or amidases to attenutate signaling. Dnr1 may inhibit Dredd activity and Caspar plays a role in the inhibition of Rel. Furthermore, activation of JNK by the dTak/Tab2 complex may lead to the recruitment of activation protein 1 (AP-1) to a repressor complex, to downregulation AMP gene transcription.

C. Signaling

The IMD adaptor, which encodes a death domain-containing protein similar to that of the mammalian tumour necrosis factor receptor (Georgel *et al*., 2001), is responsible for integrating the signals from PGRP-LC and –LE to initiate downstream signaling events. Subsequently, IMD interacts via its Death Domain (DD) with dFADD (Naitza *et al.,* 2002) and in turn dFADD is able to recruit Dredd, a caspase 8 homologue, to the complex via an interaction between their Death Effector Domains (DEDs) (Hu and Yang, 2000), although the precise mechanism of Dredd activation is not clear. Furthermore, it is not clear how the individual components of the complex are recruited to the plasma membrane from their basal localisation in the nucleus (Gomez-Angelats and Cidlowski, 2003; Screaton *et al.,* 2003; Boyer *et al.,* 2011). Whatever the precise mechanism of signal transduction, these events culminate in the phosphorylation and cleavage of the transcription factor Relish by Dredd (Ertürk-Hasdemir *et al.,* 2009). *In vitro* studies using S2 cells has revealed that a novel gene termed *Sickie* is also required for Relish cleavage, potentially supporting Relish activation by Dredd (Foley and O'Farrell, 2004). Relish itself is composed of an N-terminal NFκB domain (Rel68) and a C-terminal ankyrin-repeat domain (Rel49) (Kleino and Silverman, 2013). Rel68, which acts as the IMD pathway transcription factor, is endoproteolytically cleaved and then able to translocate to the nucleus and initiate the transcription of target genes, such as the AMPs *Drc* and *Dpt* (Stöven *et al.,* 2000).

In addition, Dredd also has a role in activation of an alternative IMD signaling pathway. Dredd has been shown to cleave IMD and thus expose a binding motif for dIAP2, a ubiquitin E3 ligase (Paquette *et al.,* 2010). This further drives the rapid polyubiquitination of IMD by other E2 ubiquitin conjugating enzymes, such as Bendless and Effete (Zhou *et al.,* 2005; Paquette *et al.,* 2010). Once ubiquitinated, IMD is able to recruit the dTak/Tab2 complex via the zinc finger domain of Tab2 (Kanayama *et al.,* 2004) and also potentially the ubiquitination linkers (Kulathu *et al.,* 2009). The activated dTak/Tab2 complex is then postulated to phosphorylate and activate the IKK complex

(Kleino and Silverman, 2013). The IKK complex itself comprises two subunits: Immune Response 5 (Ird5), a catalytic subunit, and Kenny, the regulatory subunit. These are required for the subsequent activation of Relish. Both subunits are required to mediate successful Relish cleavage (Silverman *et al.,* 2000; Stöven *et al.,* 2003). However, for complete phosphorylation, hence activation of Relish and successful target gene transcription *in vitro*, Ird5 activity must remain intact (Ertürk-Hasdemir *et al.,* 2009). Whether this link holds true for *in vivo* scenarios remains to be validated.

Whilst dTak/Tab2 has been implicated in Relish cleavage, the complex has also been perceived to play a role in the activation of Jun-N-terminal Kinase (JNK) via the JNK-kinase Hemipterous (Kleino and Silverman, 2013). However, the role of JNK within the IMD pathway is somewhat controversial. Some reports have cited that JNK plays the role of a negative regulator, mediating the recruitment of Activator Protein 1 (AP-1) to a repressor complex; to dampen down AMP gene expression upon chronic immune signaling (Kim *et al.,* 2007). In contrast, other studies have shown that JNK signaling is integral for AMP gene induction (Delaney *et al.,* 2006). Hence, with conflicting evidence, further research is required to resolve the true role of JNK within the IMD signaling pathway.

Despite the need for clarification on the precise nature of some of the molecular mechanisms behind aspects of IMD signaling, it has been well documented that the result of these combined signaling cascades is a systemic immune response that is generated on an extremely rapid timescale to a bacterial stimulus. For instance, the ubiquitination of IMD, as well as the phosphorylation and subsequent translocation of Relish to the nucleus, occurs within minutes after the initial bacterial stimulus (Paquette *et al.,* 2010). As such, the expression of target AMP genes peaks within a timescale of hours after stimulus (Lemaitre *et al.,* 1997; Vodovar *et al.,* 2005; Valanne *et al.,* 2007).

Whilst IMD signaling is acknowledged to be rapid, there are also mechanisms by which signaling can be attenuated or inhibited. Defense repressor 1 (Dnr1)

has been postulated to physically interact with and inhibit Dredd activity (Foley and O'Farrell, 2004; Guntermann *et al.,* 2009). Whilst the precise manner in which this inhibition is mediated remains elusive, Dnr1 mutations have been shown to lead to enhanced IMD signaling and increased AMP expression in the fly brain upon infection (Cao *et al.,* 2013). Another negative IMD modulator, Caspar, has been demonstrated to inhibit the cleavage of Relish by Dredd and hence attenuate the transcription of AMP genes, both in the presence and absence of a bacterial stimulus (Kim *et al.,* 2006), suggesting that it is required for constitutive pathway activation.

1.3.2 The Toll Pathway

A. Pathway Overview

The Toll pathway, an evolutionarily conserved cascade between *Drosophila* and the Toll-like pathway in mammalian systems, is triggered by a variety of fungal and Gram-positive bacterial pathogens (as reviewed in Lemaitre and Hoffmann, 2007). Recognition of invading pathogens via factors such as Lysine (Lys)-type peptidoglycan and β-1,3-glucan by PGRPs, as well as Gram Negative Bacteria-Binding Proteins (GNBPs), leads to the cleavage and subsequent activation of the extracellular cytokine Spatzle (Spz). This in turn activates the transmembrane receptor Toll, ensuring the receptor's own dimerisation (Lemaitre and Hoffmann, 2007). As a result, Pelle Kinase is recruited and in turn induces degradation of Cactus, the *Drosophila* equivalent of IκB, although the precise mechanism by which this is achieved remains elusive (Lemaitre and Hoffmann, 2007; Valanne *et al.,* 2011). Consequently, the Dorsal and Dif transcription factors are released from their complex with Cactus and are able to translocate to the nucleus and activate the transcription of target genes, such as *Drs* and *Def* (Lemaitre and Hoffmann, 2007). It has been shown that loss of function or deletion of any Toll components causes an immune deficient phenotype, including a typical lack of *Drs* expression, leading to increased susceptibility to Gram-positive and fungal infections (Rutschmann *et al.*, 2002). In contrast to IMD pathway signaling, that of the Toll pathway is often more chronic, with activation of the pathway within hours

of recognition of a bacterial stimulus and the transcription of effector genes perpetuated for several days (Lemaitre *et al.,* 1997).

B. Recognition

In terms of pathogen recognition and activation of Toll signaling, it is postulated that three distinct upstream cascades may be induced, depending on the activating microbe (Figure 1.3). In the major cascade, Gram-positive bacterial species and fungal cells are respectively recognized via their Lys-type PGN or β-1,3-glucan present in their cell walls (Valanne *et al.,* 2011). A range of PGRPs and GNBPs are able to recognize these moieties. GNBP3 has been demonstrated to be responsible for recognition of yeast species via binding to β-1,3-glucan (Gottar *et al.,* 2006), whereas PGRP-SA, PGRP-SD and GNBP-1 seem to more specifically recognize Lys-type PGN (Valanne *et al.,* 2011). Upon the recognition of Lys-type PGN, PGRP-SA and GNBP-1 form a complex (Michel *et al.,* 2001; Gobert *et al.*, 2003; Pili-Floury *et al.,* 2004). However, the precise mechanism of how this event then translates into initiation of the subsequent signaling cascade remains elusive. Some evidence suggests that forming this complex activates GNBP-1 to hydrolyse the PGN to produce glycan-reducing ends, which are subsequently presented to PGRP-SA (Wang *et al.,* 2006). Other studies suggest that this is not the case as GNBP-1 appears to lack enzymatic activity, and in fact may play a role as a linker between PGRP-SA and the remainder of the signaling cascade (Buchon *et al.,* 2009). Little is also known about the role of PGRP-SD in Toll pathway activation; it appears to function as a receptor for both Gram-positive and Gram-negative bacteria (Leone *et al.,* 2008), with some partial redundancy to the PGRP-SA-GNBP-1 complex (Bischoff *et al.,* 2004).

Fungal and Gram-positive bacterial recognition signaling is subsequently integrated by Modular Serine Protease (modSP); this protease provides the link between recognition and a signaling cascade consisting of the serine proteases Grass, Spirit, Spheroide and Sphinx 1/2 (Kambris *et al.,* 2006; Buchon *et al.,* 2009). The end result of this serine protease cascade is that Spatzle-processing enzyme (SPE) is activated and Spz, the ligand for core

Toll signaling, is processed to its active form (DeLotto and DeLotto, 1998; Jang *et al.,* 2006). Spz is secreted in an inactive precursor form, consisting of a C-terminal region and a prodomain (DeLotto and DeLotto, 1998). The prodomain shields the hydrophobic C-terminal region (Arnot *et al.,* 2010) and activation via prodomain cleavage induces a conformational change that results in the hydrophobic regions being exposed and available for binding to the Toll receptor (Arnot *et al.,* 2010). Thus far, two models concerning the binding of Spz to the Toll receptor have been postulated; firstly, it was suggested that one Spz dimer binds a dimerised pair of Toll receptors (Weber *et al.,* 2005), but a newer model implies that two Spz dimers may bind to a Toll receptor dimer, with one Spz pair binding to each N terminus of the receptor (Gangloff *et al.,* 2008). Currently, nine Toll receptors have been characterised in *Drosophila*, with all sharing a similar molecular structure comprising a Leucine-rich ectodomain with Cysteine-rich flanking motifs (Valanne *et al.,* 2011). Toll-1 is the isoform which thus far has shown to be responsible for the production of AMPs (Valanne *et al.,* 2011), but Toll-5 and Toll-9 may also play roles, having been previously implicated in *Drs* and *Mtk* expression (Imler *et al.,* 2000; Tauszig *et al.,* 2000; Luo *et al.,* 2001; Ooi *et al.,* 2002).

As mentioned previously, other upstream signaling cascades also leads to the activation of Spz; one such pathway is facilitated by the protease Persephone (Psh). Psh is able to detect virulence factors from fungal and Gram-positive bacterial species, which leads to its proteolytic maturation, after which Psh is able to activate SPE leading to the activation of Toll receptors in the manner described above (Gottar *et al.,* 2006; El Chamy *et al.,* 2008). It should also be noted that Toll signaling plays a critical role in development, as well as being an immune signaling cascade. Dorsal-ventral patterning. Thus, in a third pathway Spz is processed into its active form by a separate serine protease cascade, including Gastrulation Defective, Snake and Easter (Chasan *et al.,* 1992; Hong and Hashimoto, 1995), with the independent sulfotransferase Pipe required for the activation of the ventral signal in the oocyte (Cho *et al.,* 2010).

C. Core Toll signaling

Upstream signaling events merge upon the cleavage and activation of Spz and its binding to the Toll receptor, thus signaling after this forms the core Toll pathway (Figure 1.4). The activated Toll receptor subsequently binds the adaptor protein MyD88 via its intracellular (TIR) domains (Horng and Medzhitov, 2001; Tauszig-Delamasure *et al.,* 2002; Sun *et al.,* 2002). Another adaptor protein, Tube, and Pelle kinase are recruited to form a heterotrimer through DD interactions (Xiao *et al.,* 1999; Sun *et al.,* 2002; Moncrieffe *et al.,* 2008), with Tube acting as a conduit between MyD88 and Pelle (Sun *et al.,* 2002). Moreover, Pellino, a Pelle/IL-1R-associated kinase interacting protein, has been shown to act as a positive regulator of Toll signaling, without which *Drs* expression is impaired (Haghayeghi *et al.,* 2010). However, the precise molecular mechanism by which this Pellino-mediated regulation is facilitated is presently unclear.

Subsequently, the inhibitory protein Cactus is phosphorylated and degraded; upon basal conditions, Cactus is bound to and thus inhibits the activity of the Toll pathway transcription factors Dif and Dorsal (Valanne *et al.,* 2011). It is not clear precisely how the phosphorylation and degradation of Cactus is mediated, but it has been speculated that this action is performed by Pelle (Towb *et al.,* 2001; Huang *et al.,* 2010) or some other so-called “Cactus kinase” which has yet to be identified. Further speculation of how this mechanism functions involves G Protein-couple reception kinase 2 (Gprk2), which was identified as a regulator of the Toll pathway in an RNAi screen using S2 cells (Valanne *et al.,* 2010) and shown to be crucial for hemocyte activation *in vivo* (Valanne *et al.,* 2011). Gprk2 interacts with Cactus in S2 cells but is not involved in its degradation (Valanne *et al.,* 2010), thus its precise function remains unknown. Nevertheless, with the consequent removal of Cactus, Dif and Dorsal are thus free to translocate to the nucleus (Wu and Anderson, 1998) and induce the transcription of target genes. In addition, another transcription factor, Deformed epiderminal autoregulatory factor 1 (Deaf1), has been determined to bind to the *Drs* and *Mtk* promoters and is required for full *Drs* expression and to effectively defend against fungal infections (Reed *et al.,* 2008).

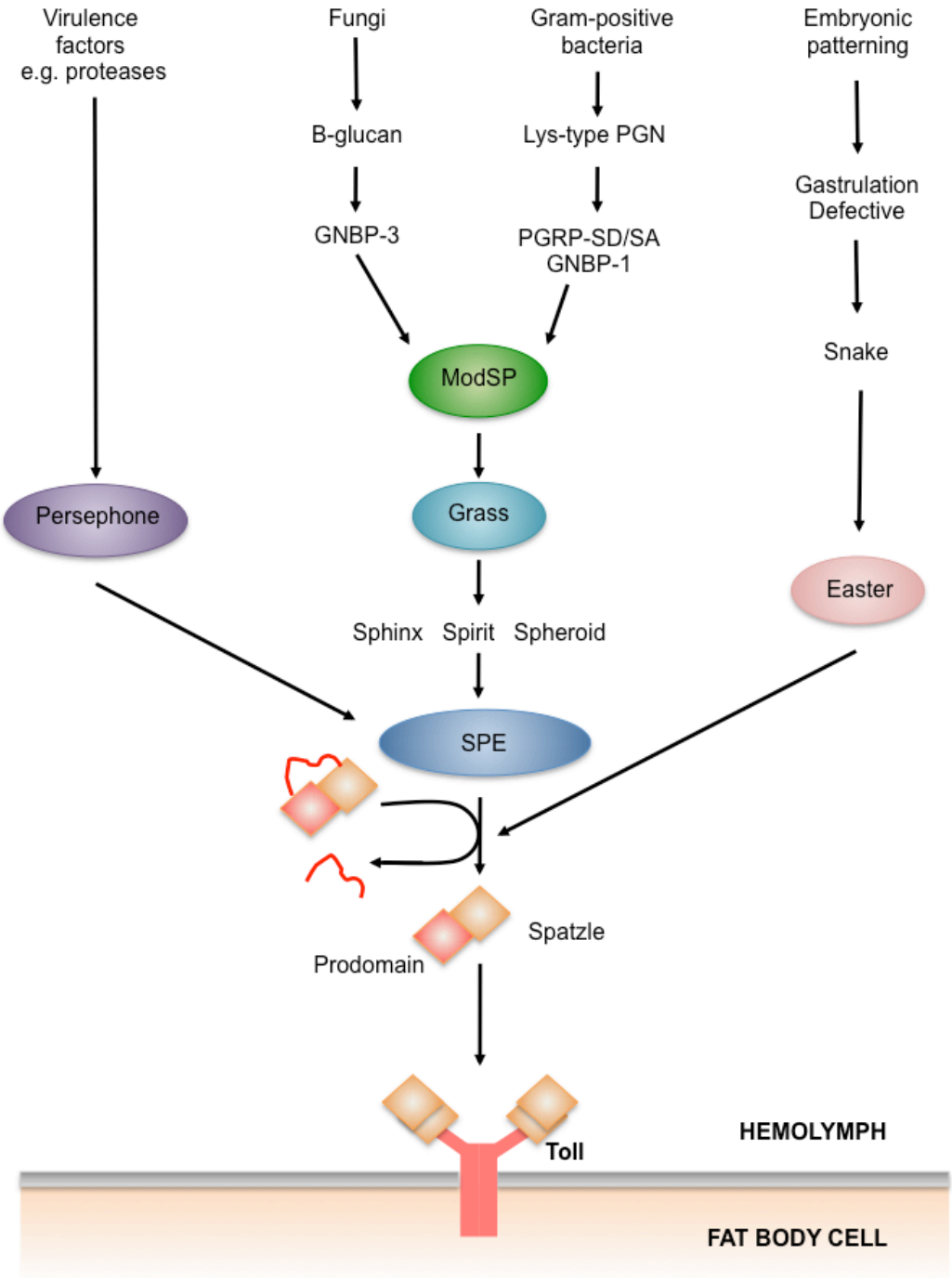

Figure 1.3: Recognition of Fungal and Gram-positive pathogens and downstream Toll signaling. Three pathways lead to the activation of Spatzle (spz), the ligand for the Toll pathway. Virulence factors, such as bacterial or fungal proteases, induce the activation of the protease Persephone, which in turn activated Spatzle-processing enzyme (SPE) to remove the prodomain of the ligand, activating Spz dimers and allowing their binding to the Toll receptor. Fungi and Gram-positive bacteria are recognized by their β-glucan and Lysine-type peptidoglycan (Lys- type PGN) via peptidoglycan recognition proteins (PGRP) SD and SA, and Gram-negative binding proteins (GNBP) 3 and 1. This event initiates a serine protease signaling cascade, involving Modular Serine Protease (modSP), Grass, Sphinx, Spirit and Spheroid. This results in the activation of SPE. Normal developmental events, such as Dorsal-Ventral patterning, also require Toll pathway signaling and this is achieve via the direct activation of Spz via a separate protease cascade involving Gastrulation Defective, Snake and Easter.

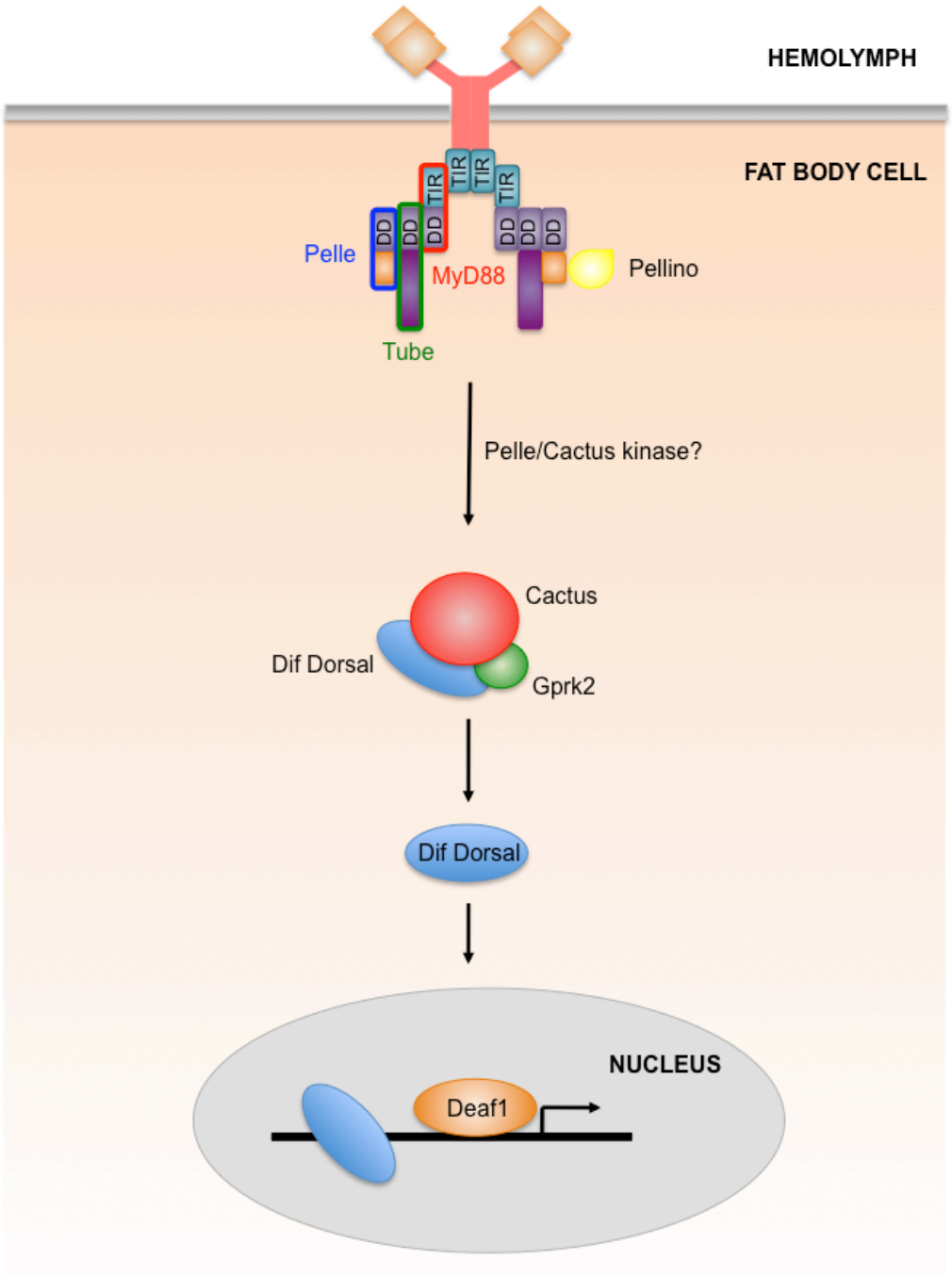

Figure 1.4: Schematic of the Core Toll Signaling Pathway. Dimerisation and hence activation of the Toll receptor leads to the recruitment of adaptor proteins MyD88 and Tube, via interactions between the intracellular (TIR) and Death Domains (DD) of the receptor and adaptors. This leads to the recruitment of Pelle kinase via DD interactions. Pellino is a positive regulator of Toll signaling via its interaction with Pelle kinase. Cactus is phosphorylated potentially by Pelle or some other yet unnamed 'Cactus kinase', thus releasing the transcription factors Dif and Dorsal from its inhibition. Grpk2 is a regulator of the Toll pathway and may associate with Cactus, although its precise function is unknown. These transcription factors are subsequently able to translocate to the nucleus and induce AMP gene expression. Deaf1 is required to induce Toll pathway genes that are downstream of the Dif/Dorsal promoter such as *Mtk* and *Drs*.

1.3.3 Cross-talk between the IMD and Toll pathways

It is also speculative whether the Toll pathway may in some manner synergise with the IMD pathway, via the formation of Relish/Dif heterodimers (Bangham *et al.,* 2006); to support this notion, *Drs* expression has been seen to receive a significant input from the IMD pathway in the systemic response (Lemaitre and Hoffmann, 2007), and as noted above is solely expressed as a result of IMD pathway activation in the local immune response. Moreover, some components may also act independently of the Toll pathway; Matskevich *et al.* (2010) have shown that GNBP-3 assembles phenoloxidase antifungal defense complexes, independent of its Toll pathway function. Therefore, it is clear that despite a wealth of knowledge concerning both the Toll and IMD pathways, that much still requires clarification in terms of precise biochemical mechanisms of pathway components, and pathway synergism.

1.3.4 The Janus Kinase /Signal Transducer and Activator of Transcription (JAK/STAT) pathway

Whilst the roles that IMD and Toll signaling play within the systemic immune response to bacterial infection have been relatively well characterized, recent research has highlighted that other signaling pathways may also contribute to this branch of the immune response. One example of these is the JAK/STAT pathway. The JAK/STAT pathway itself consists of a receptor, Domeless, which is activated by three closely related cytokine-like ligands, which show homology to human interleukins; these are termed Unpaired, Unpaired 2 and Unpaired 3 (Upd, Upd2 and Upd3) respectively (Agaisse *et al.,* 2003), as depicted in Figure 1.5 Upon ligand binding, Domeless subsequently signals to a JAK termed Hopscotch, resulting in the dimerization and ensuing activation of the transcription factor STAT92E (Lemaitre and Hoffmann, 2007). This complex can then translocate to the nucleus to induce gene transcription.

The JAK/STAT pathway was originally identified as a potential immune pathway via JAK/STAT deficient flies; this line showed a particular sensitivity

to the *Drosophila C* virus (Dostert *et al*., 2005). Moreover, in the *Anopheles* mosquito model, it has been shown that after immune challenge with *E. coli* or *M. luteus* that the corresponding STAT component relocated to the nuclei of fat body cells (Barillas-Mury *et al.,* 1999). In parallel, *Drosophila* models demonstrated that Upd3 and STAT were expressed in the gut after challenge with the bacterium *Serratia marcescens* (Cronin *et al.,* 2009). Consequently, in *Drosophila* the activation of this pathway was shown to regulate the expression of a subset of immune-responsive genes, predominately the *Turandot* stress genes and the gene encoding the complement-like protein *Tep2* (Lemaitre and Hoffmann, 2007). The *Turandot* (*tot*) genes currently have no specifically assigned function, but numerous stress conditions, in particular septic injury, have been shown to be pivotal to their induction (Levy *et al*., 2004).

However, it has also been suggested that activation of JAK/STAT can lead to proliferation (Buchon *et al.,* 2009), whereby stem cell proliferation is induced via the JAK/STAT pathway through the release of Upd3 from damaged cells. Buchon *et al*. (2009) observed this phenomenon in enterocytes of *Drosophila* adults infected with *Erwinia carotovora carotovora 15 (Ecc15)*, a Gram-negative bacterium, highlighting the role of JAK/STAT in the regulation of epithelial renewal upon infection. Moreover, Pastor-Pareja *et al.* (2008) verified that all three Upd isoforms were upregulated in mechanically wounded wing discs and tumours induced in *Drosophila* larvae. As a result of ligand secretion, a systemic amplification of the response was observed, with JAK/STAT signaling shown to be activated in hemocytes in response to wounds and tumours, which was subsequently required for their proliferation (Pastor-Pareja *et al.,* 2008). Thus, it has been postulated that the JAK/STAT pathway could respond to tissue damage, but it should be noted that the exact role this pathway plays within the total host defense remains to be determined (Lemaitre and Hoffmann, 2007).

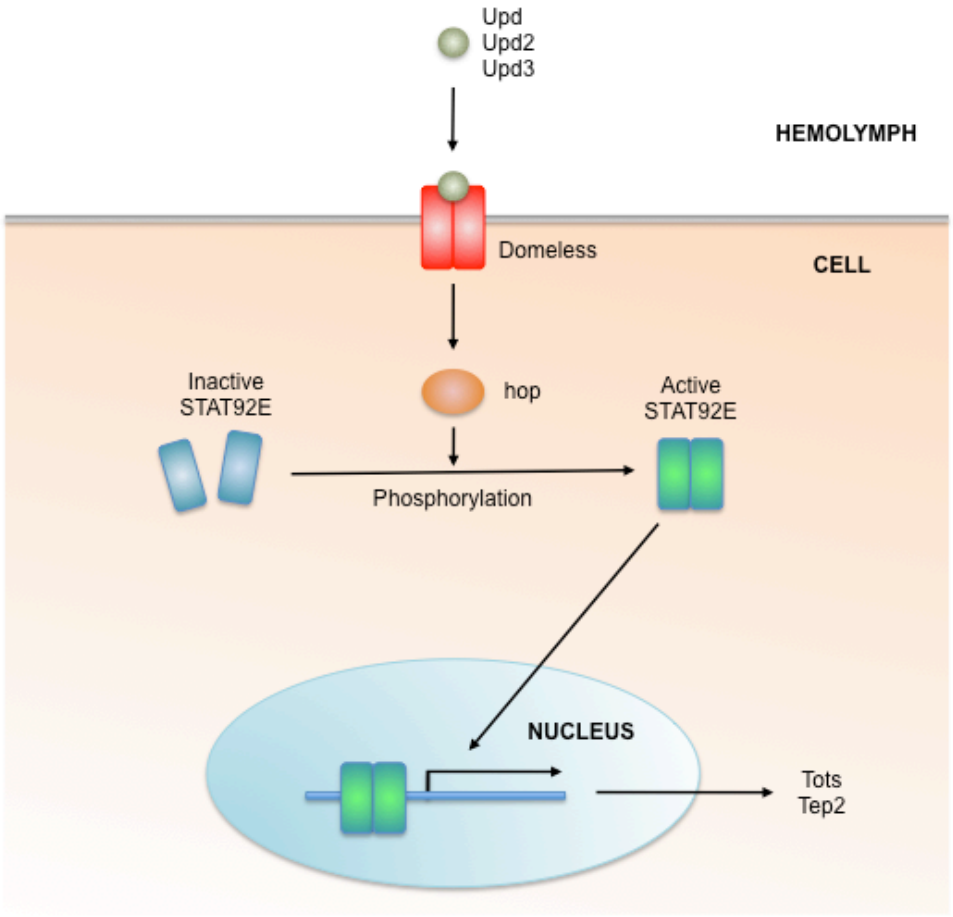

Figure 1.5: An Overview of the JAK-STAT pathway in *Drosophila*. The receptor Domeless is activated by its cytokine-like ligand, Upd3, which leads to the recruitment and activation of Hopscotch (hop), a JAK. This subsequently leads to the activation of STAT92E via phosphorylation and dimerisation, and STAT92E dimers translocate to the nucleus to induce the transcription of gene products, such as those of the *Turandot (tot)* and *Tep2* genes.

1.3.5 The JNK pathway

Aside from its role in IMD signaling, where it has been postulated to induce the expression of AMPs (Kallio *et al.,* 2005), JNK signaling has previously been demonstrated to regulate not only many *Drosophila* developmental processes, but also processes that are required for damage resolution. For instance, JNK signaling is necessary for proper wound healing of the epidermis (Lemaitre and Hoffmann, 2007) and upregulation of JNK activity has also been observed as a result of tissue damage following the induction of cancerous tumours or wounding in *Drosophila* larval discs (Pastor-Pareja, *et al.,* 2008). In terms of

infection, dTak1 has been shown to activate Basket, the *Drosophila* equivalent of JNK, in response to bacteria (Boutros *et al.,* 2002). Subsequently, JNK-dependent immune genes have also been shown to have a role in cytoskeleton remodeling, perhaps suggesting a role in hemocyte activation (Boutros *et al.,* 2002). Furthermore, infection of the gut with *C. albicans* is able to promote high levels of gut cell death; a phenomenon that was partially attributed to activation of JNK signaling by fungal proteases (Glittenberg *et al.,* 2011), suggesting that infection has a direct effect on JNK signaling in *Drosophila*. Thus, whilst it is clear that infection has a significant impact on JNK signaling, and in particular highlighting the complex nature of cross-talk within *Drosophila* systemic immune, more research is required to clarify its precise role within the overall immune response in *Drosophila*.

1.3.6 Other proposed pathways and signaling mediators

Aside from the key pathways described above, other novel modulators of the immune response have been identified, some of which represent novel mechanisms of regulating innate immunity, independent of IMD and Toll signaling. For instance, the transcription factor of the insulin-like growth factor signaling cascade, forkhead box subgroup O (FOXO), which is a key regulator of the stress response to starvation (Mattilla *et al.,* 2009), adapting metabolism to suit nutrient conditions (Hafen, 2004) and longevity and ageing (Vermeulen and Loeschcke, 2007), has also been demonstrated to regulate AMP activation independent of both the IMD and Toll pathways (Becker *et al.,* 2010). This FOXO-driven AMP activation could be induced in non-infected larvae upon starvation and was speculated to aid in the modulation of the immune defense, particularly when the host may be suffering from energy shortage or stress. Moreover, this mechanism of FOXO-dependent AMP regulation is conserved between *Drosophila* and mammalian systems (Becker *et al.,* 2010). The authors propose that this would represent a cross-regulation of metabolism and immunity, whereby AMPs could potentially be induced under basal physiological conditions in response to an oscillating degree of energy within cells and tissues, independent of pathogen recognition.

Another novel example of IMD and Toll-independent immune regulation was presented by Junell *et al.* (2010); the authors cited that the transcription factor Ventral veinless (Vvl) plays a role in tissue-specific regulation of innate immunity in *Drosophila*, driving the expression of *Cec*, again independently of the Toll and IMD pathways. It was subsequently proposed that Vvl acts in concert with other as of yet unknown regulators to control constitutive AMP gene expression in a tissue-specific fashion, particularly in immune-competent tissues that are continuously exposed to pathogens (Junell *et al.,* 2010). However, no further mechanism concerning how Vvl may be driving AMP expression has become apparent. Hence, whilst knowledge regarding the traditional systemic immune signaling pathways is undoubtedly extensive in *Drosophila*, it must also be acknowledged that it is somewhat incomplete and continually expanding, with novel mechanisms described that have not as of yet been successfully incorporated into the existing paradigm.

Other examples of novel signaling pathways that may synergise with traditional immune signaling pathways come from *in vitro* studies with S2 cells. Niermann-Pick disease type C2a (NPC2a) and Niermann-Pick disease type C2e (NPC2e) proteins are induced in response to infection with both *E. coli* and *S. aureus* in *Drosophila* larvae and adult flies (Shi *et al.,* 2012). Moreover, these proteins were then shown to activate the *Dpt* promoter in S2 cells when stimulated with either Lys- or DAP-type PGN respectively (Shi *et al.,* 2012). However, the authors of Shi *et al.* (2012) do not give any hypothesis for how NCP2a and NCP2e may be directly interacting with the established components of IMD pathway to regulate AMP expression. Hence, even when novel mechanisms can be demonstrated to interact with the established immune signaling machinery, the precise details of how this occurs can remain elusive.

1.4 Epithelial responses

1.4.1 Local immune Response

An area of the immune response on which relatively little information is known is the so-called local immune response. Separate from both the systemic and cellular responses, this genre of immune response involves the production of AMPs localized to the surface epithelia of specific tissues which are in continual contact with the external environment (Ferrandon *et al.,* 1998; Tzou *et al.,* 2000), such as the gut, trachea, reproductive tract and epithelium of *Drosophila* (Ferrandon *et al.,* 2007). This expression can be classed as either constitutive or inducible (Lemaitre and Hoffmann, 2007). For instance, constitutive *Drs* expression has been noted in the salivary glands (Tzou *et al.,* 2000), and *Cec* in the male ejaculatory duct (Tzou *et al.,* 2000). This expression is regulated by various tissue specific transcription factors, such as Caudal (Ryu *et al.,* 2004; Han *et al.,* 2004), in contrast to the NFκB-like transcription factors of the systemic Toll and IMD pathways. Inducible local AMP expression is triggered by infection with Gram-negative bacteria and mediated by the IMD pathway (Tzou *et al.,* 2000; Tingvall *et al.* 2001); for example, both Drosomycin and Diptericin have both been observed to be induced in the trachea and gut via the IMD pathway as a result of local infection (Basset *et al.,* 2000). However, with this is mind, it remains unclear why commensal bacteria of the gut or ingested PGN do not induce chronic immune activation in these epithelia (Ha et al., 2009a). One explanation might be the scavenging ability of amidase PGRPs, which may sequester PGN released by commensal organisms within the gut. Upon infection, PGN titres may reach a threshold level above the scavenging capabilities of these receptors and thus allow differentiation between commensal and pathogen presences (Lemaitre and Hoffmann, 2007). No role for Toll pathway activity in the local immune response has been demonstrated thus far; moreover, there is no data to suggest that AMPs are induced in epithelia in response to Gram-positive bacteria or fungi (Ferrandon *et al.,* 1998; Lemaitre and Hoffmann, 2007).

1.4.2. Reactive Oxygen Species Production

In mammalian systems, the generation of reactive oxygen species (ROS) is an immediate response to infection; a so-called “respiratory burst”, resulting in the production of superoxide and hydrogen peroxide and subsequently hypochlorous acid, a highly bactericidal compound (Molina-Cruz *et al.,* 2007). This is also the case in *Drosophila*, where infection induces rapid ROS production in the gut (Ha *et al.,* 2005a; Ha *et al.,* 2005b; Ha *et al.,* 2009a), which may also have microbicidal effects. One protein that is vital in the generation of ROS is Dual oxidase (Duox) (Ritsick *et al.,* 2004), which demonstrates NADPH oxidase activity (Anh *et al.,* 2011). When *Duox* is inactivated in infected flies via RNAi, ROS production is severely inhibited, suggesting that this enzyme is the unique source of epithelial ROS (Ha *et al.,* 2005b). Moreover, *Duox-RNAi* flies are highly and rapidly susceptible to oral infection of *Erwinia carotovora* (Ha *et al.,* 2005b; Nehme *et al.,* 2007). The host protection conferred by Duox was subsequently demonstrated to be modulated via phospholipase C-β, which mobilized intracellular calcium required for ROS production via Duox (Ha *et al.,* 2009). In addition to ROS-mediating killing of microbes, it has subsequently been observed that the production of ROS, such as hydrogen peroxide, is also pivotal in the recruitment of hemocytes to areas of damage in *Drosophila* (Moreira *et al.,* 2010) via calcium-mediated activation of Duox (Razzell *et al.,* 2013).

On the other hand, an excessive and prolonged ROS response is potentially harmful to any host, causing oxidative stress, loss of cell function and eventually apoptosis or necrosis (Nordberg and Arnér, 2001). This scenario is prevented in *Drosophila* by the enzyme Immune Responsive Catalase (IRC). Evidence has demonstrated the importance of this enzyme, as silencing IRC by RNAi not only results in enhanced ROS production, but also increased fly mortality (Ha *et al.,* 2005b). Overall, this is indicative that IRC forms the reciprocal half of a *Drosophila* antioxidant defense system; where a fine balance between IRC and Duox activity is required for control of microorganisms. This is seemingly critical for microbial control in the gut lumen, where it provides an additional defense barrier against ingested pathogens, independent of IMD signaling (Ryu *et al.,* 2006).

1.5 The Cellular Immune Response

1.5.1 Hemocyte classification

The cellular response is composed of a collection of innate immune mechanisms mediated by a specific group of immune cells termed hemocytes. Although three categories of hemocyte are recognized within the current literature (Lemaitre and Hoffmann, 2007), the predominant hemocyte sub-group is undoubtedly the plasmatocyte, which represents 90-95% of all mature larval hemocytes (Lemaitre and Hoffmann, 2007); these are professional phagocytes (Jiravanichpaisal *et al.,* 2006) and the equivalents of mammalian monocyte-macrophage lineage cells (Williams, 2007). This sub-group of hemocytes patrols adult and larval *Drosophila* tissues, engulfing apoptotic debris and microbial pathogens (Lanot *et al.,* 2001; Wood and Jacinto, 2007). At the pupal stage they play a crucial role by both engulfing and recycling superfluous cells during metamorphosis (Lanot *et al.,* 2001), as well as providing an immune surveillance system to prevent infection by bacterial species that normally results in pupal lethality (Charroux and Royet, 2009). Plasmatocytes are also critical in the clotting process in response to mechanical injury; plasmatocytes release hemolectin and other proteins that subsequently form clot fibres (Lemaitre and Hoffmann, 2007). In *Drosophila* hematopoiesis, mature plasmatocytes arise in two distinct phases: one in the head mesoderm during embryogenesis and the second in the lymph gland during larval development (Tepass *et al.,* 1994; Evans *et al.,* 2003; Holz *et al.,* 2003; Meister, 2004). The plasmatocytes derived from embryogenesis persist into larval stages, forming the mature circulating hemocytes of this stage of development. In contrast, the lymph-derived hemocytes do not circulate until metamorphosis under non-immune conditions (Stofanko *et al.,* 2010).

The remaining 5% of total *Drosophila* hemocytes consist largely of crystal cells, which play a critical role in the defense-related melanization process to sequester and kill invading microorganisms (Jiravanichpaisal *et al.,* 2006). Much rarer are the lamellocytes, which are involved in the encapsulation and neutralization of objects too large to be effectively phagocytosed, such as

parasitoid eggs (Lemaitre and Hoffmann, 2007). Plasmatocytes, crystal cells and lamellocytes are postulated to arise from different lineages of cells originating from a common pro-hemocyte stem cell (Lanot *et al.,* 2001; Meister, 2003), with differentiation into the distinct hemocyte cell types influenced by exposure to differential signaling and transcription factors (Qiu *et al.,* 1998; Alfonso and Jones, 2002; Zettervall *et al.,* 2004; Bataille *et al.,* 2005). Other models suggest that lamellocytes may actually arise from plasmatocytes (Sorrentino *et al.,* 2007; Frandsen *et al.,* 2008; Stofanko *et al.,* 2010). Interestingly, it must be noted that, in contrast to plasmatocytes, crystal cells and lamellocytes have no clear mammalian homologues (Meister, 2004).

Thus whilst hemocytes all ultimately arise from a common pro-hemocyte progenitor (Lanot *et al.,* 2001), the roles that the different sub-types of hemocytes play within the immune response are distinct and diverse. These will now be examined in greater detail.

1.5.2 Opsonisation

Opsonisation is the process whereby foreign organisms or particles are marked for destruction; typically, pathogens are coated in phagocytosis markers, termed opsonins, which allow their recognition and consequent phagocytosis (Owens III and Peppas, 2006). In vertebrate systems, opsonins are represented by immunoglobulins and components of the complement system (Nakanishi and Shiratsuchi, 2006). However, it is unclear whether opsonisation-dependent phagocytosis is active in invertebrate systems, such as *Drosophila* (Nakanishi and Shiratsuchi, 2006), although recent studies have identified some potential opsonin candidates (Lagueux *et al.,* 2000; Stroschein-Stevenson *et al.,* 2006). Lagueux *et al.* (2000) determined that the *Drosophila* genome was demonstrated to contain six genes coding for proteins that are structurally related to the complement α2-macroglobulin family. Of these, five contain a thioester motif and hence are designated as Thioester-containing proteins (Tep1-5) (Lagueux *et al.,* 2000). Tep6, otherwise known as Macroglobulin-complement related (Mcr), is structurally distinct, as it lacks a

specific cysteine residue that normally leads to the formation of the characteristic Tep thioester (Lagueux *et al.,* 2000). Of these proteins, Tep5 does not appear to be actively expressed (Lagueux *et al.,* 2000). In terms of localisation, Teps have been shown to be actively expressed in many immune tissues, such as hemocytes, barrier epithelia and can be induced in the fat body (Bou Aoun *et al.,* 2010). Furthermore, Teps were shown to be basally expressed but upregulated upon infection in adult flies and larvae (Lagueux *et al.,* 2000; De Gregorio *et al.,* 2001; Irving *et al.,* 2005; Dionne *et al.,* 2006). Tep2, Tep3 and Tep4 have been suggested to play a role in the opsonisation and subsequent phagocytosis of *E. coli, S. aureus* and *C. albicans* respectively (Nakanishi and Shiratsuchi, 2006; Stroschein-Stevenson *et al.,* 2006). Tep1 is also upregulated upon infection (Lagueux *et al.,* 2000), which is not necessarily surprising given its homologue in the mosquito *Anopheles gambiae* plays a role in the killing of the parasite *Plasmodium* (Blandin *et al.,* 2004). Moreover, Mcr was shown to be secreted by S2 cells and able to bind to *C. albicans* with high affinity (Stroschein-Stevenson *et al.,* 2006). Thus, it has been postulated that Teps may function as opsonins during the immune response to promote phagocytosis, or alternatively as protease inhibitors (Lemaitre and Hoffman, 2007). Conversely, other studies have shown that members of the Tep family, such as Tep1, 2 and 4, are not required in the body cavity of adult flies in order to eliminate a range of bacterial and fungal infections, suggesting some form of redundancy or compensation by other elements of the immune response (Bou Aoun *et al.,* 2010).

1.5.3 Phagocytosis

Phagocytosis is the process where cells internalize particles (Chung and Kocks, 2011) and is crucial for the elimination of apoptotic bodies and invading pathogens; it is an ancient mechanism that is evolutionarily conserved between *Drosophila* and mammalian systems (Rabinovitch, 1995; Williams, 2007). In *Drosophila*, this process is mediated by the plasmatocytes (Williams, 2007). In terms of infection, for phagocytosis to proceed successfully, receptors on the surface of the plasmatocyte must recognize an invading

pathogen. To carry out this function, these receptors either recognize their targets directly or via opsonins (Wright *et al.,* 1989). Of the former category, studies have uncovered a variety of candidate receptor proteins that are required for efficient phagocytosis, including pathogen recognition receptors (Kocks *et al.,* 2005; Ulvila *et al.,* 2006). One such protein is the Scavenger receptor SR-CI, a pattern recognition receptor that binds to bacteria (Rämet *et al.,* 2001). Other scavenger receptors include Peste, a CD36 family member which is required for uptake of *Mycobacterium fortuitum* (Philips *et al.,* 2005), and Eater, which is expressed by hemocytes and required to survive infections with both Gram-negative and Gram-positive bacteria (Kocks *et al.,* 2005; Chung and Kocks, 2011). Eater may also cooperate with AMP expression, with studies elucidating that CecA promoted Eater binding to *E. coli* (Chung and Kocks, 2011). In terms of candidate phagocytosis receptors, Williams (2007) cites the immunoglobulin superfamily protein Down syndrome cell adhesion molecule (Dscam) as one of the most promising potential targets, due to its ability to bind bacteria to promote effective phagocytosis (Watson *et al.,* 2005) and the potential for its 18000 alternative splice variants produced in hemocytes and fat body cells to form a diverse range of pathogen recognition receptors (Watson *et al.,* 2005; Williams, 2007). Moreover, screens in S2 cells have identified further proteins that are involved in phagocytosis of a variety of bacterial species, such as *E. coli* and *S. aureus* (Ramet et al., 2002; Pearson *et al.,* 2003). These include cytoskeletal proteins, such as SCAR, an activator of the Arp2/3 complex which regulates actin filament nucleation (Machesky *et al.,* 1999; Welch and Mullins, 2002; Zallen *et al.,* 2002), Nimrod C1, a member of the Nimrod family of proteins with EGF-like repeats (Kurucz *et al.*, 2007), and PGRP-LC (Ramet *et al.,* 2002).

However, the precise mechanisms by which some elements of phagocytosis occur still remain relatively elusive; for example, although candidate *Drosophila* receptors for phagocytosis by plasmatocytes, such as Draper, a protein containing a NIM repeat that has been shown to play a role in the phagocytosis of *S. aureus* and apoptotic cells (Freeman *et al.,* 2003; Manaka *et al.,* 2004; Cuttell *et al.,* 2008), and Nimrod C1, a transmembrane protein with EGF-like repeats (Kurucz *et al.,* 2007), have been reported, how they

directly or indirectly recognize bacteria still remains to be clarified (Hashimoto *et al.,* 2009). Some progress has recently been made to address this with the discovery that lipoteichoic acid may act as a bacterial ligand (Hashimoto *et al.,* 2009), and Pretaporter as a *Drosophila* apoptotic ligand (Kuraishi *et al.,* 2009) for the receptor Draper.

1.5.4 Melanisation

Melanisation is an immediate immune response observed at a site of cuticle injury or on the surface through which parasites invade the hemocoel (Lemaitre and Hoffmann, 2007). This response is thought to play an important function in wound healing, sequestration of microorganisms and the production of toxic intermediate molecules that are able to kill invading pathogens (Ashida, 1990; Nappi and Vass, 1993; Söderhäll and Cerenius, 1998). At sites of injury in *Drosophila* larvae, crystal cells solely mediate the melanisation response (Lemaitre and Hoffmann, 2007). This is evident in flies which exhibit abnormal crystal cells, such as *Black cells* mutants (Lemaitre and Hoffmann, 2007), or those that completely lack crystal cells altogether, such as *lozenge* mutants (Rizki *et al.,* 1980); in these mutant backgrounds, melanisation is severely impaired.

The melanisation reaction involves the *de novo* synthesis and deposition of melanin, resulting in a blackening of tissue. A schematic of the responsible signaling cascade is shown in Figure 1.6. Whilst it is not explicitly known what initiates the cascade in *Drosophila*, studies in other insect models inferred that injury or recognition of microbial ligands, such as PGN, β-(1,3)-glucan, and LPS, through pattern recognition receptors may initiate the signaling cascade (Ochiai and Ashida, 1999; Ochiai and Ashida, 2000; Ma and Kanost, 2000; Lee *et al.,* 2004). In one part of the signaling cascade, the enzyme prophenoloxidase (PPO) must be activated to its activated phenoloxidase (PO) form; this enzyme is then rapidly released from crystal cells and able to catalyse the oxidation of mono and diphenols to orthoquinones to form melanin (Lemaitre and Hoffmann, 2007). To achieve this PPO is cleaved by

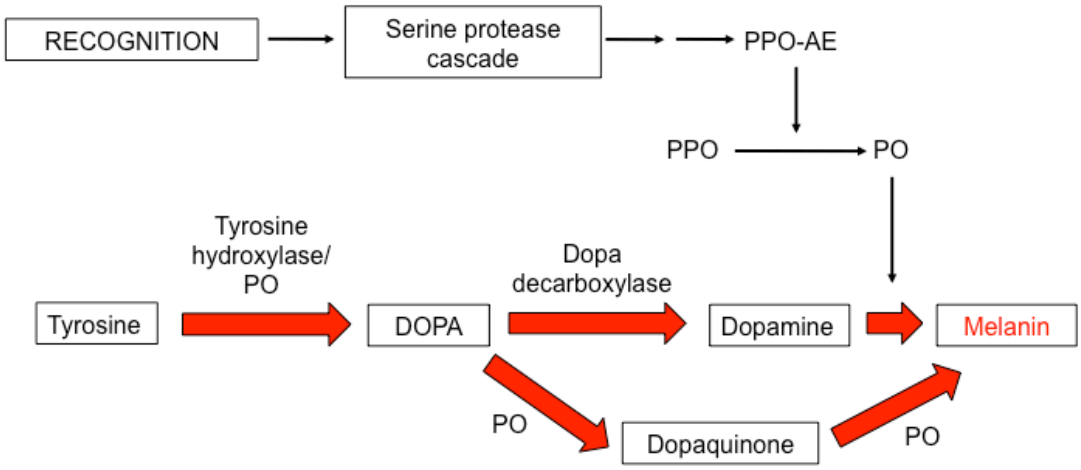

Figure 1.6: Schematic of the Melanisation Reaction. Bacterial ligands are recognized and, via serine protease signaling, prophenoloxidase-activating enzyme (PPO-AE) converts prophenoloxidase (PPO) to its active phenoloxidase (PO) form. PO can then, along with tyrosine hydroxylase convert tyrosine to 3,4-dihydroxyphenylalanine (DOPA). DOPA is processed to dopamine by dopa decarboxylase or to dopaquinone by PO. PO subsequently converts these two substrates to melanin, via a series of intermediary steps.

PPO-activating enzyme (PPO-AE). Data from other insect models has suggested that PPO-AE itself exists as a zymogen that is activated by a serine protease signaling cascade, initiated by the recognition of microbial ligands (Ashida and Brey 1995; Jiang *et al.,* 1998). The remaining strand of the melanisation reaction involves the conversion of tyrosine to melanin via quinone intermediates. Tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase or PO (Tang, 2009). DOPA can then be converted either to dopamine by dopa decarboxylase (ddc) or dopaquinone by PO itself (Tang, 2009). Finally, dopamine and dopaquinone are both converted via several intermediate steps into melanin by PO (Tang, 2009). Whilst melanin is thought to sequester microbes at wound sites, other quinone and ROS intermediates, such as dopaquinone, are postulated to kill microorganisms at this location (Christensen et al., 2005). There is also recent evidence to suggest that other signaling pathways may play a role in mediating the melanisation response. For instance, daw was demonstrated to have a role in the inhibition of melanisation; *daw* knockdown flies exhibited melanotic tumours and increased expression levels of serine protease 7 (Sp7), an enzyme which is specifically required for infection-induced melanisation

(Clark *et al.,* 2011). Subsequently, experiments where *daw* was overexpressd in adult fat body demonstrated that daw suppressed Sp7 infection-induced melanisation, as well as inhibiting *Sp7* expression in the absence of any infection (Clarke *et al.*, 2011).

Conversely, there has previously been dispute over the importance of melanisation in combating infection (Leclerc *et al.,* 2006; Tang *et al.,* 2006). Leclerc *et al.* (2006) demonstrated that flies unable to activate prophenoloxidase were not any more or less susceptible to infection with a variety of fungal and bacterial species than wild-type controls, proposing the notion that melanisation as a whole may not have any significant role in immunological defense. However, it must also be acknowledged that this study assessed the response of prophenoloxidase mutant flies to a relatively limited range of pathogens. Ayres and Schneider (2008) conducted infection studies with a much broader range of bacterial pathogens, concluding that some bacterial infections, such as *Salmonella typhimurium*, induced a disseminated melanisation response in the fly and that absence of a functional phenoloxidase gene greatly impacted on *Drosophila* resistance and tolerance capabilities to such infections. The importance of melanisation to the *Drosophila* immune response can also be inferred from other insect models, such as the mosquito *Anopheles gambiae*, where the melanisation response has been implicated to be key in the immune response to infection with entomopathogenic fungus (Yassine *et al.,* 2012) and the parasitic protozoan *Plasmodium* (Volz *et al.,* 2006). Thus, it is clear that melanisation may play an important role in the *Drosophila* immune response.

1.5.5 Encapsulation

Encapsulation is the process by which lamellocytes protect against invading parasites in the *Drosophila* larval stage (Lemaitre and Hoffmann, 2007). Wasp eggs implanted in larvae are detected by circulating plasmatocytes (Russo *et al.,* 1996), and also elicits the release of plasmatocytes from the lymph gland (Lanot *et al.,* 2001) and also potentially from the sessile larval population

(Zettervall *et al.,* 2004). Regardless of their origin, these plasmatocytes subsequently attach to the egg chorion, forming septate junctions and effectively separating the egg from the hemocoel (Russo *et al.,* 1996; Williams et al., 2005). This action, through a currently unknown mechanism, causes a massive differentiation of lamellocytes within the lymph gland (Jung *et al.,* 2005). These lamellocytes differentiate from pro-hemocytes originating from the medullary zone and secondary lobes (Carton and Nappi, 1997; Meister and Lagueux, 2003; Jung *et al.,* 2005). The lamellocytes are subsequently released from the lymph gland and gather around the egg, forming a multi-layered capsule structure (Lemaitre and Hoffmann, 2007). It is within this capsule that the wasp egg is eventually eliminated, potentially by the local production of toxic products, such as ROS (Nappi *et al.,* 1995). The capsule is often also accompanied by melanisation, thus intermediate products of the melanisation cascade may also play a crucial role in mediating wasp egg killing (Nappi *et al.,* 1995). Despite speculation, the precise causes of parasite death, as well as the particular molecular mechanisms behind the whole process of encapsulation, are not known (Lemaitre and Hoffman, 2007).

However, whilst many elements of the encapsulation process remain unspecified, Williams (2007) has proposed that this process must be dependent upon adhesion and cell shape change. Thus it has been suggested that integrins may function in this process; it has been shown that mutant alleles of *myospheroid*, a β-integrin subunit gene, reduce the efficiency of capsule formation with lamellocytes failing to attach to the wasp egg (Irving *et al.,* 2005). Also, it has been speculated that members of the Rho GTPase protein family, which are responsible for the regulation of cell migration and shape change (Barrett *et al.,* 1997; Paladi and Tepass, 2004), may also play a role in regulating encapsulation. Rac GTPases Rac1 and Rac2 are required for the effective encapsulation of parasitoid wasp eggs (Williams *et al.,* 2005; Williams *et al.,* 2006). Moreover, in Rac2 mutant larvae plasmatocytes and lamellocytes have been demonstrated to attach to the target wasp eggs but fail to spread around it leading to a subsequent failure in capsule melanisation (Williams, 2007). Rac 1 and Basket are also crucial for proper encapsulation,

but the reason for this requirement remains unknown (Williams *et al.,* 2006). Conversely, the depletion of Hemese, a transmembrane glycophorin-like protein present on hemocytes and hematopoietic organs, leads to an enhanced encapsulation response, which suggests that it plays a role in the activation or recruitment of hemocytes (Kurucz *et al.,* 2003). Finally, microarray studies have determined that Toll and JAK/STAT signaling is activated in response to parasitization and are required for an effective encapsulation response (Sorrentino *et al.,* 2002; Wertheim *et al.,* 2005), although the precise roles of these pathways in the encapsulation process are not defined.

1.5.6 Coagulation

Coagulation has many roles within *Drosophila* larvae upon septic injury. It firstly blocks entry of invading pathogens into the hemocoel, working to seal wounds and trap microbes (Theopold *et al.,* 2002; Theopold *et al.,* 2004). This response must be rapid, to ensure that fluid loss is minimized, especially in larvae where the hemolymph is under great pressure to form the hydroskeleton (Bidla *et al.,* 2005). As a result, a clot is rapidly generated at the site of wounding in *Drosophila* larvae; this predominantly comprises fibres of hemolectin, a multi-domain protein with similarity to other invertebrate and vertebrate clotting factors, which traps circulating plasmatocytes at the wound site (Goto *et al.,* 2001). Other proteins that are present in the clot have been assessed via proteomic studies (Scherfer *et al.,* 2004; Karlsson *et al.,* 2004). One such protein is Fondue, a Toll-pathway regulated hemolymph component, without which clotting defects are observed (Scherfer *et al.,* 2006); Fondue is required for the cross-linking of clot fibres. However, wounding of larvae and flies deficient in *fondue* or *hemolectin* does not necessarily lead to increased mortality compared to controls (Scherfer *et al.,* 2004; Scherfer *et al.,* 2006), although some studies would also suggest that this is not always the case (Chang *et al.,* 2012). Other clot proteins include lipophorin, larval serum proteins and fat body protein 1 (Karlsson *et al.,* 2004; Scherfer *et al.,* 2004). Whilst these proteins clearly function in clot assembly to some degree, their

precise roles have not been clearly defined. Further questions remain unanswered in terms of the contribution that melanisation may make to this process; coagulation is considered as a process independent of melanisation, yet some components of the melanisation cascade such as PPO may be responsible for the hardening of clots (Karlsson *et al.,* 2004; Bidla *et al.,* 2005; Bajzek *et al.,* 2012), and have been shown to form part of the clot matrix (Karlsson *et al.,* 2004; Scherfer *et al.,* 2004). Furthermore, this response requires the cooperation of plasmatocytes, which have been shown to secrete hemolectin (Theopold *et al.,* 2013), and crystal cells which contain PPO (Theopold *et al.,* 2013), but the mechanism by which this, and that of the activation of the hemocytes themselves, is mediated remains unknown. It is possible that wound cues may play a role in this process.

1.5.7 Cross-talk with the Systemic Immune Response

Research has also indicated that the cellular branch of the *Drosophila* immune response may also synergize with the systemic branch, with plasmatocytes providing a positive feedback to the fat body in order to further promote the systemic immune response and subsequent production of AMPs in *Drosophila* larvae (Agaisse *et al.,* 2003; Charroux and Royet, 2009). Charroux and Royet (2009) determined that adult flies depleted of plasmatocytes, mediated by the expression of the proapoptotic protein Hid specifically in hemocytes, demonstrate a strong susceptibility to infection despite wild-type expression levels of AMP genes. This strongly suggests that a deficit in one branch of the *Drosophila* immune response cannot necessarily be compensated for by the activities of the other.

In terms of the biological mechanism by which cross-talk between the systemic and cellular response may be mediated, Shia *et al.* (2009) demonstrated that the secretion of Spz by hemocytes was required for Toll-dependent AMP gene transcription in the larval fat body. This finding would suggest some involved of the Toll pathway in mediating such cross-talk in *Drosophila*. Furthermore, Brennan *et al.* (2007) established that *psidin*, which

encodes a lysosomal protein that is required by plasmatocytes to successfully degrade engulfed bacteria, was also required for *Def* expression in the fat body. This study subsequently proposed a model whereby hemocytes were able to activate AMP production by the fat body upon antigen presentation by hemocytes (Brennan *et al.,* 2007). Whilst this study provided a crucial link between pathogen detection by the cellular response and subsequent AMP production by the fat body, as Kounatidis and Ligoxygakis (2012) note, the authors fail to address that the rescue of *psidin* mutants was performed by expressing wild-type psidin using the *peroxidasin-Gal4* driver, which could also lead to expression in fat body tissue. Therefore, there may also be a requirement for *psidin* in both tissues, despite the authors' claim that this is restricted to plasmatocytes (Kounatidis and Ligoxygakis, 2012). Nevertheless, it is clear that interaction between both the systemic and cellular responses in *Drosophila* is required for effective immunity, with both branches working in concert to provide protection against microbial challenge (Elrod-Erickson *et al.,* 2000), although further work is required to precisely elucidate the nature of this relationship.

However, other work has led to the proposition that such a connection is limited or does not exist. Elrod-Erickson *et al.* (2000) demonstrated that inhibiting the hemocyte phagocytic capacity, via the introduction of polystyrene beads within these cells, did not confer significant susceptibility to *E. coli* infections in wild-type flies (Elrod-Erickson *et al.,* 2000); only preventing phagocytosis in *imd/imd* mutant flies had any effect on fly survival to *E. coli* infection (Elrod-Erickson *et al.,* 2000). This would in turn indicate that phagocytosis, and hence this area of the cellular response, may play a relatively minor role in the overall host defense of *Drosophila*, and in the balance of cellular versus systemic immune branch activity. Moreover, it is known that flies with a defective cellular immune response can be rescued from the effects of Gram-positive infections via the strengthening of the systemic immune response via the overexpression of AMP genes using the Gal4UAS system (Nehme *et al.,* 2011), again suggesting that the involvement of the cellular response to the overall host defense is not fundamentally required, in contrast to the results of Charroux and Royet (2009). However, it

must be noted that these studies focused solely on plasmatocytes and their phagocytic capacities, and thus their conclusions may not be representative of interactions of all elements of the cellular immune response with the systemic branch. Therefore, it is clear from the limited and conflicting knowledge presented that the precise nature and mechanism of systemic and cellular response interaction is not well characterised.

1.6 The Immune Response to Damage

Recent studies have demonstrated that an immune response may not be generated in response to challenge by pathogens alone; in fact, damage signaling may also play a role in the activation of immune mediators such as AMPs. This is not a particularly novel concept in itself; it has been previously documented that in mammalian systems when no microbial stimulus is present, a T-cell immune response is generated (Kono and Rock, 2008). This may be triggered as a response to tissue transplants, tumours and autoimmune disease (Kono and Rock, 2008). As a result, Matzinger (1994) proposed a concept whereby the immune system could respond to damage, stress or non-physiological cell death; the so-called “Danger Hypothesis”. This concept highlighted how abnormal cell death could be recognized by hallmarks termed damage-associated molecular patterns (DAMPs) and thus could be interpreted as a universal sign of danger and potentially stimulate immune responses (Matzinger, 1994).

However, it is only relatively recently that any empirical evidence has corroborated this hypothesis, particularly in *Drosophila* and regarding the innate immune response. For instance, several genes have been demonstrated to be expressed by hemocytes at a wound site generated by sterile laser ablation, including *phospholipase A1*, and *Drs* (Stramer *et al.,* 2008). Furthermore, Buchon *et al.* (2009) demonstrated that stress and cell damage induced in the gut of adult flies by *Erwinia* infection resulted in the activation of JAK/STAT signaling and the expression of *Dro3*. Other validations of the danger hypothesis in *Drosophila* include the observation that JNK signaling is activated by tissue damage in tumours and aseptic wounds,

leading to the expression and amplification of JAK/STAT activating cytokine signaling by the fat body and hemocytes (Pastor-Pareja *et al.,* 2008). Localised DNA damage was also shown to induce a systemic response in *Drosophila* larvae by Karpac *et al.* (2011). UV irradiation of 2nd instar larvae triggered the expression of many AMP genes, including AttB, AttC and Def, in the fat body via the IMD pathway (Karpac *et al.,* 2011). Moreover, this response is limited by insulin/IGF signaling and that a balance between these two signaling pathways is required to coordinate proper growth, metabolic activities and the response to damage (Karpac *et al.,* 2011). Therefore, when taking these results into account, it is possible that an immune response may be activated independently of microbial stimulation; hence, the immune response itself may be considered a response to damage/non-damage as opposed to a response to self/non-self. However, it is not yet entirely clear how some of these responses are mediated, and the extent of interplay between the immune responses stimulated following damage or microbial recognition.

1.7 *Drosophila* embryos as a Model of Infection

Despite the success of *Drosophila* as an immunity model, the limits of this model are appreciable. For example, the outcome of infection experiments is often measured by insect death or changes in cell morphology at specific time-points throughout infection. As a result, crucial information on the early stages of infection, particularly early host-pathogen interactions, has previously been unattainable, due to the lack of ability to observe infections in real-time. Due to the use of adult *Drosophila* and larvae in previous immunity studies, a real-time imaging approach has until recently been considered non-viable. A study by Vlisidou *et al.* (2009) addressed this issue by using *Drosophila* embryos to study the early stages of infection with *Photorhabdus asymbiotica* and *Escherichia coli* via time-lapse confocal microscopy, elucidating much novel information; for example, that embryonic hemocytes were able to recognize and phagocytose non-pathogenic *E. coli* and that *P. asymbiotica* infection led to a “frozen” hemocyte phenotype, with a significant reorganization of the actin

cytoskeleton (Vlisidou *et al.,* 2009). Moreover, Stramer *et al.* (2008) further used transgenic *Drosophila* embryos containing reporter constructs to assess gene, particularly AMP, induction following sterile wounding; showing upregulation of *Drs* in the fat body, yolk sack and hemocytes of the embryo. Thus, although a relatively novel model of immunity and until recently overlooked in favour of adult or larval models, *Drosophila* embryos have already demonstrated their ability to generate new insights into immune activation and regulation, relieving some of the potential caveats of previous studies. However, due to the lack of immunity studies using *Drosophila* embryos, and despite their development being well documented (Hartenstein, 1993), the ability of embryos to activate immune responses to infection remains largely unknown.

1.8 Experimental Aims

The central aim of this project was to develop a more detailed and intricate model of bacterial infection using the *Drosophila* embryo, and to assess its potential as a viable immunity model. Initial aims concentrated on establishing whether *Drosophila* embryos were able to mount an effective AMP response to various types of infection, and whether this was mediated via the canonical *Drosophila* systemic immunity pathways. Moreover, a parallel aim was to determine if multiple facets of the cellular and systemic response were intact and functional within the embryo system, and behaved in a manner which mirrored the host defense of the adult fly and larval models. In addition to this initial characterization, a further key aim of this project was to determine at what stage of development *Drosophila* embryos begin to demonstrate immune competence and what biochemical signaling pathways may drive this immunological phenomenon.

Having established the immune capacity of the embryo in terms of its ability to respond to bacterial challenge, subsequent project objectives focused on the determination of global transcriptome changes within the embryonic system upon infection with Gram-negative or Gram-positive microbes, or sterile

damage induced by either laser ablation or sterile injection procedures. This would enable the identification of the novel genes that were modulated in *Drosophila* upon infection or damage. Another analogous aims was to determine the precise role of hemocytes upon either infection or damage within the *Drosophila* embryo; to monitor the transcriptional changes within this particular cell type when faced with microbial challenge or sterile damage. Ultimately, the aim of this section of the project was to determine if any hemocyte-specific infection or damage genes are present in *Drosophila*.

1.9 Project Strategy

To address these aims, the project strategy initially consisted of a candidate approach, whereby live infection studies were combined with molecular biology techniques. *Drosophila* embryo lines containing immune gene reporter fusions were used to initially assess embryo immune capabilities. Wild-type and immune mutant fly lines were used to assay survival upon bacterial infection. Quantitative polymerase chain reaction methodologies were then used to determine the relative levels of immune gene expression upon treatment with a range of different bacterial species.

To determine the transcriptional changes within the whole embryo and specifically in hemocytes upon infection, microarray studies were conducted. These were combined with Fluorescence Assisted Cell Sorting (FACS), in order to isolate hemocytes for RNA processing. By determining which genes were modulated within the embryo upon infection or damage stimuli, induced via microinjection of microbes or sterile laser ablation of embryos respectively, it would be possible to assess the total infected or wounded transcriptomes of the *Drosophila* embryo. Furthermore, it enabled the investigation of whether any novel genes are modulated within the embryo upon infection or damage. Combined with the transcriptome data from specifically hemocytes which had been in contact with infection stimuli, this approach allowed the possibility of identifying the existence of hemocyte-specific infection genes. Further

networking analysis was then employed to uncover further trends and patterns within the expression data.

Chapter 2 – Materials and Methods

1. Materials

1.1 General laboratory reagents

Halocarbon 700 oil, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sodium phosphate, potassium phosphate, 100% heptane, 100% ethanol, 100% chloroform, 36% formaldehyde, mowiol, 20-hydroxyecdysone (5mg), 1,4-diazabiycylo[2.2.2]octane, TritonX-100, Bovine Serum Albumin, Goat Serum and Schneiders media without Calcium Chloride were purchased from Sigma-Aldrich (Dorset, UK). Phosphate buffered saline (PBS) tablets were purchased from Oxoid Ltd. (Cambridge, UK). Diethylpyrocarbonate (DEPC)-treated water and endotoxin-free water were obtained from Cambio (Cambridge, UK). 25mM dNTP mix was purchased from Promega (Madison, USA). Neat commercial bleach (<5% sodium hypochlorite) was purchased from The Consortium (Coventry, UK).

1.2 Specific materials

Qiazol, the Rneasy® Mini Kit and Dneasy® Blood and Tissue Kit were purchased from Qiagen (West Sussex, UK). The Superscript III First Strand Synthesis System, The Qubit™ Quantification Platform and Quant-iT™ assay kit were obtained from Invitrogen (Paisley, UK). TurboDNase (2U/μL), 10X Dnase Buffer, DNase Inactivation Reagent, RNA later and the RNAqueous MicroKit were purchased from Ambion (Austin, USA). Trypsin (100mg), collagenase (100mg) and protease from *Aspergillus oryzae* were obtained from Sigma-Aldrich (Dorset, UK). Nylon cell strainers (70μm) for embryo collection and for hemocyte FACS experiments (40μm) were ordered from BD Biosciences (Oxford, UK). UVette protein-free cuvettes, FemtoJet capillaries and microloader tips were purchased from Eppendorf (Stevenage, UK). Borosilicate tissue grinders and pestles were obtained from Fisher Scientific

(Loughborough, UK). 24x32cm Knittel-Glaser coverslips, 22x22cm and 18x18cm glass coverslips of thickness 1 were purchased from Scientific Laboratory Supplies Ltd. (Nottingham, UK). PetriPerm hydrophobic membranes for embryo wounding assays were procured from Pure Proteomics (company dissolved). The Experion Automated Electrophoresis System, Experion RNA StdSens Analysis Kit and RNA ladder, qPCR plates, iTaq SYBR Green supermix and StepOne Real-Time PCR Detection System were obtained from Biorad (California, USA). Nunclon cell culture dishes for hemocyte extraction and imaging were purchased from Fisher Scientific (Loughborough, UK). Primary antibodies were obtained from the Developmental Studies Hybridoma Bank (DSHB; Iowa City, USA) or Abcam (Cambridge, UK). Further details can be found in Table 2.6. Secondary antibodies were obtained from Invitrogen (Paisley, UK).

Apple juice agar plates, embryo glue and fly food were manufactured in house, the constituents of which can be located in Appendix 1. Embryo glue was manufactured from double sided Scotch tape and 100% heptane. Tungsten needles were also manufactured in house, using tungsten wire bought from Goodfellow (Huntington, UK).

1.3 Bacterial culture materials

The antibiotics Spectomycin, Rifampicin, Ampicillin and Carbenicillin were purchased from Sigma-Aldrich (Dorset, UK). For Luria Bertani (LB) media, tryptone, yeast extract and sodium chloride for microbial media were obtained from Fisher Scientific (Loughborough, UK). For LB agar, plant agar was ordered from Sigma-Aldrich (Dorset, UK). LB media and agar were generated and autoclaved in house.

1.4 Fly stocks

A. Gal4 drivers

The Gal4 fly stocks displayed in Table 2.1 were used to drive expression of UAS constructs within embryos.

Table 2.1: Gal4 Fly Stocks used to Drive UAS Construct Expression

Fly stock	Source	Reference
w; breathless Gal4 (w; btlGal4)	Bloomington Stock Centre	Shiga *et al.,* 1996
w;; croquemort Gal4 (w;;crqGal4)	Bloomington Stock Centre	Stramer *et al*., 2005
w;; daughterless Gal4 (w;;daGal4)	Bloomington Stock Centre	Wodarz *et al.,* 1995.
w; engrailed^{E22c} Gal4 (w; E22cGal4)	Bloomington Stock Centre	Lawrence *et al.,* 1995
w; serpentGal4 (w; srpGal4)	Bloomington Stock Centre, Indiana	Bruckner *et al.,* 2004

B. Fluorescent UAS constructs

Fly stocks containing transgenic UAS constructs driving fluorescent protein expression in Table 2.2 were used to observe hemocyte morphology, dynamics and phagocytic capabilities.

Table 2.2: Fluorescent UAS construct fly lines used

Fly stock	Source	Reference
w; uas GFP	Bloomington Stock Centre	Yeh *et al*., 1995
w;; uas GFP	Bloomington Stock Centre	Yeh *et al*., 1995
w; uas moesin cherry	Gift from P. Martin	Millard and Martin, 2008

C. Other UAS constructs

Other transgenic UAS constructs (Table 2.3) were expressed in hemocytes to assess the effect of specific genes on hemocyte motility and responses to bacteria stimulation.

Table 2.3: Other UAS fly lines used

Fly stock	Source	Reference
w; uas Ecdysone Receptor -BI Dominant Negative (w; uas EcR-B1 DN)	Bloomington Stock Centre	Cherbas *et al*., 2003

D. GFP fusion lines

The GFP fusion lines below (Table 2.4) were used to study the localisation and time-course of the immune response either within embryos post bacterial infection or post wounding via sterile laser ablation.

Table 2.4: GFP fusion lines used for infection experiments

Fly stock	Source	Reference
w; Drocosin-GFP (w; Drc-GFP)	Gift from J.L. Imler	Tzou *et al.,* 1998.
w; Drosomycin-GFP (w; Drs-GFP)	Gift from B. Stramer.	Ferrandon *et al.,* 1998.
w; Peptidoglycan recognition protein – LC – GFP (w; PGRP-LC-GFP)	Gift from B. Lemaitre	Schmidt *et al.,* 2008

E. Mutants

Table 2.5 illustrates the mutants lines employed: *Relish*^{E20} (*Rel*^{E20}), *modular serine protease* (*modSP*^{1}) and double *Persephone*^{1} (*psh*^{1}) and *modSP*^{1} mutant lines were homozygous for their mutations and hence had no requirement for balancers. *Ecdysone receptor* (*EcR*) ^{Q50st}, *EcR*^{M554fs} and *Scar*$^{\Delta 37}$ were all balanced using *CTG*. In these cases, selecting embryos that

did not contain the *CTG* balancer allowed for homozygous mutants to be procured.

Table 2.5: Immune mutant lines used

Fly stock	Mutation type	Source	Reference
w; Rel^{E20}	Loss of function	Gift from B. Lemaitre	Hedengren *et al.,* 1999.
w;; modSP^{1}	Amorph	Gift from B. Lemaitre	Buchon *et al.,* 2009.
w, psh^{1};;modSP^{1}	Amorph	Gift from B. Lemaitre	Buchon *et al.,* 2009.
w; EcR^{Q50st}	Amorph	Bloomington Stock Centre	Bender *et al.,* 1997.
w; EcR^{M554fs}	Amorph	Bloomington Stock Centre	Bender *et al.,* 1997.
w; SCAR$^{\Delta 37}$	Amorph		Zallen *et al.,* 2002.

The list of fly lines generated from the above stock lines can be found in Appendix 2.

1.5 Bacterial strains

Erwinia carotova carotova 15 (*Ecc15*) was obtained from B. Lemaitre, EPFL, Lausanne, Switzerland. *Micrococcus luteus*, *DH5α Escherichia coli*, BL-21 *RFP-Escherichia coli*, *4134 Salmonella typhimurium* and *TTO1 Photorhabdus luminescens* strains were obtained from N. Waterfield, University of Bath, UK. *Mg1655 Escherichia coli* were obtained from the American Type Culture Collection, Virginia, USA.

1.6 Antibodies

Tables 2.6 and 2.7 display the primary and secondary antibodies utilized for immunofluorescent staining. Primary antibodies were diluted in 40% glycerol to make concentrated stocks. Working stocks were subsequently generated by diluting to the required concentration with 0.1% Triton X-100, 1% BSA in PBS or 0.1% Triton X-100, 10% Goat Serum in PBS, dependant on requirement.

Table 2.6: Primary antibodies

Antibody	Raised in	Epitope recognised	From	Dilution
Anti-GFP	Rabbit	GFP	Abcam	1:500
Anti-2A12	Mouse	Tracheal lumen protein	DSHB	1:5
Anti-EcR-common	Mouse	Common domain in all EcR isoforms	DSHB	1:25

Table 2.7 Secondary antibodies

Antibody	Raised in	Fluorescence	From	Dilution
Anti-rabbit	Goat	Alexa Fluor 488	Molecular probes	1:500
Anti-mouse	Goat	Alexa Fluor 568	Molecular probes	1:500
Anti-mouse IgM	Goat	Alexa Fluor 568	Molecular probes	1:500

2. Methods

2.1 Fly stock maintenance and amplification

All *Drosophila* lines were maintained at the University of Bath, UK at 22°C, under standard conditions, on food consisting of yeast extract, malt extract, soya flour, corn meal, plant agar and sugar. To amplify fly stocks, vials of flies were tipped onto new food in vials every 2-3 days until enough progeny had emerged from old containers to establish a laying cage or for use in a genetic cross.

2.2 Fly Collection and Genetic Crosses

Fly crosses were established at 22°C with a minimum of 10 flies. Flies were transferred into a vial of standard food with a drop of yeast paste to encourage mating. The flies were tipped on to new food and yeast every 2-3 days until a sufficient number of progeny were obtained. These progeny were then in turn utilized in further crosses or transferred to laying cages.

2.3 Laying cages

To generate *Drosophila* embryos for studies, laying cages of flies were established (Figure 2.1). At least 20 female and 20 male flies of the appropriate genotypes were anaesthetized using CO_2 and transferred to a plastic beaker with holes in the bottom. An apple juice agar plate with a drop of yeast paste attached to the side was placed to seal the beaker and secured using an elastic band. The beaker was inverted once flies had recovered. The laying cages were incubated at 22°C in darkness for 15-16 hours to promote mating and embryo deposition onto the agar plates. The agar plates could then be removed to harvest the embryos.

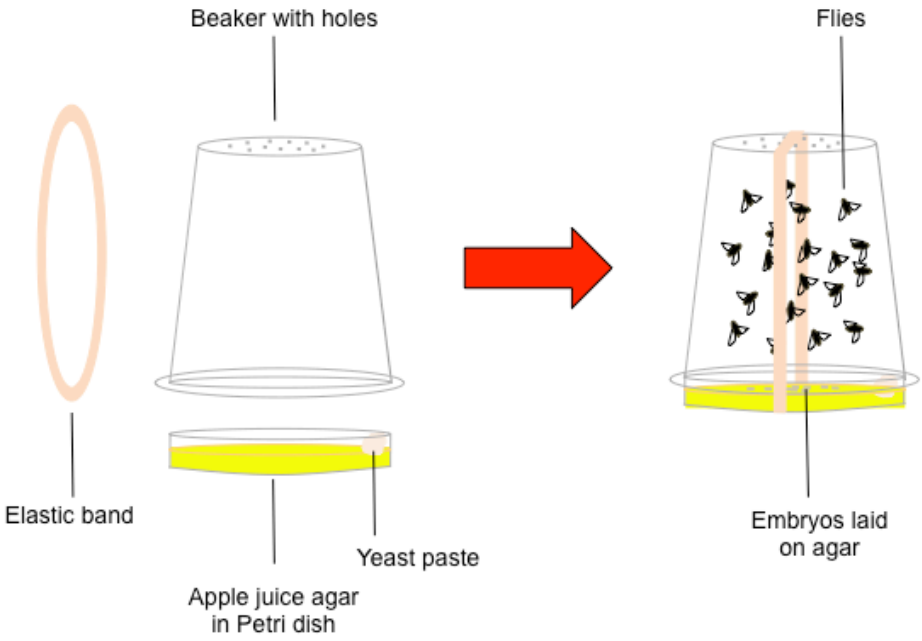

Figure 2.1 Schematic of a Laying Cage. To establish a laying cage to procure *Drosophila* embryos, a drop of yeast paste was added to the side of an apple juice agar plate. Flies were then anaesthetized using CO_2 and placed inside a beaker with holes. The agar plate was then used to seal the beaker and the whole apparatus held together using an elastic band. Cages would be stored on their side until the flies recovered at which point they would be stored as depicted in the schematic. Flies subsequently laid embryos on the agar, which could be removed to harvest embryos for experiments.

2.4 *Drosophila* embryo preparation

Embryos were transferred to a 70μm nylon cell strainer by brushing into distilled water using a paintbrush and dechorionated by immersing into neat commercial bleach for 2 minutes. To remove any traces of bleach, the embryos were rinsed 5 times with distilled water in the strainer and then kept immersed in distilled water until required.

2.5 Mounting

A. For imaging

Embryos of the appropriate stage of development were selected from the cell strainer using a tungsten needle (Figure 2.2A), in accordance with the Atlas of *Drosophila* Development (Hartenstein, 1993). Embryos were then placed on double sided tape adhered to a glass slide and orientated so their ventral side

was exposed. Approximately 5-7 embryos were mounted per slide. Upon completing this task, two 22x22mm glass coverslips were adhered to the tape on either side of the embryos. Minimal Halocarbon 700 oil was dabbed on top of the embryos using a tungsten needle to protect against desiccation. A 24x32mm glass coverslip was then carefully lowered to rest on top of the flanking coverslips using tweezers and secured using nail polish.

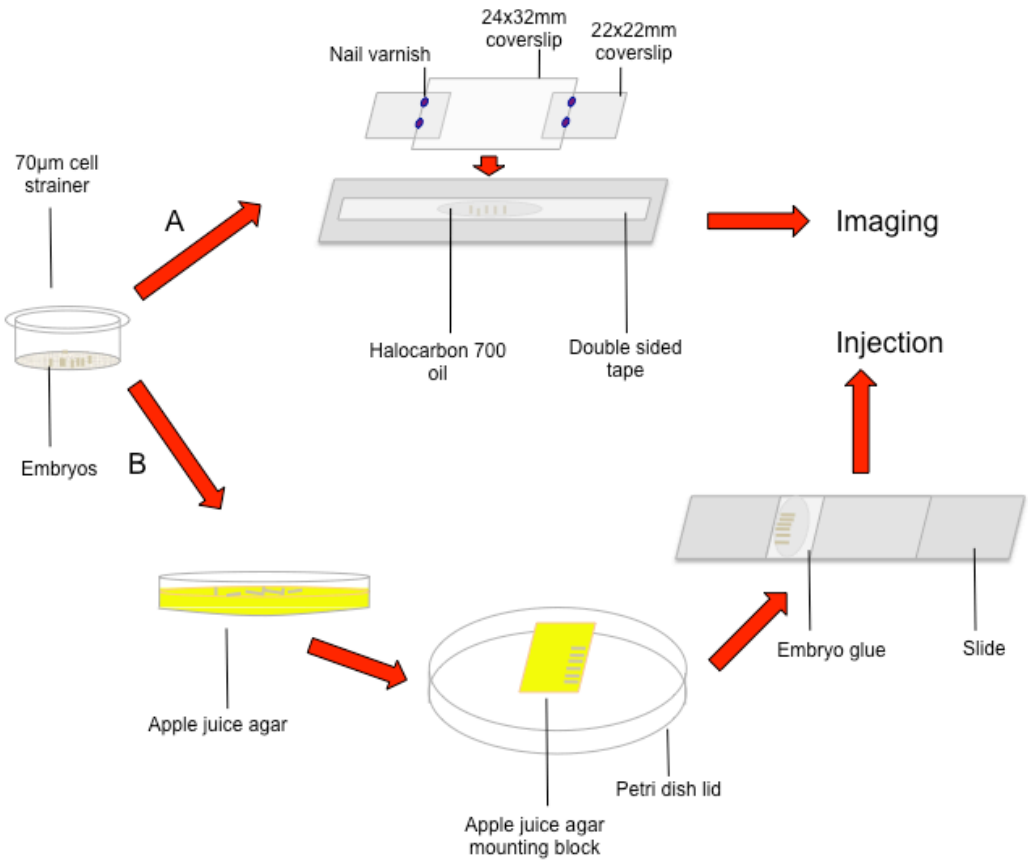

Figure 2.2: Procedures for mounting *Drosophila* embryos. Embryos were mounted either for imaging (A) or microinjection (B). A: Embryos of the correct developmental stage were individually selected directly from the cell strainer onto double sided tape, covered with halocarbon oil and a coverslip bridge placed and secured on top. B: Embryos were transferred to an apple juice agar plate where those of the correct developmental stage were collected and transferred as a group to an agar mounting block. Here they were lined up, transferred to a line of glue on a coverslip and mounted on a slide for microinjection.

B. For microinjection

All embryos were transferred from the cell strainer to an apple juice agar plate using a paintbrush (Figure 2.2B). Embryos of the correct developmental stage were selected and grouped as above until required. These embryos were then transferred to a mounting block; a square piece of apple juice agar on a Petri dish lid. The embryos were lined parallel to the edges of the agar, with no more than 25 embryos per line. Embryos were orientated so that their ventral side was in contact with the agar and so that their anterior was facing towards the middle of the block. The resulting lines of embryos were lifted from the block by placing a 24x32mm coverslip covered with embryo glue on top. The coverslips were then adhered to a glass slide using a drop of Halocarbon 700 oil and desiccated for 8 minutes in a box of silica salt. Upon completing desiccation, the line of embryos was covered with halocarbon 700 oil, to prevent further embryo desiccation, and the slide stored in a box in humid conditions for 10 minutes prior to injection.

2.6 Bacterial culture and preparation

All cultures were isolated from 40% glycerol stocks stored at -80°C by streaking onto sterile LB agar plates, with antibiotic where appropriate as detailed in Table 2.6. One colony was then selected and sub-cultured overnight in 10mL LB media in a sterile falcon tube under the conditions detailed in Table 2.6 and with antibiotic where appropriate.

To prepare live bacteria for infection experiments, 1mL of culture was washed three times in sterile endotoxin-free PBS. The optical density of the culture at 600nm (OD_{600}) was measured using a 1:10 dilution of the original culture in endotoxin-free PBS in an Uvette cuvette using an Eppendorf Biophotometer. The OD_{600} of the washed bacterial was then readjusted using sterile endotoxin-free PBS to the required OD as detailed in Table 2.7. To prepare heat killed bacteria for infection assays, 1mL of bacterial culture was washed three times in sterile endotoxin-free PBS as above and resuspended in sterile-endotoxin free PBS to the required OD_{600} value. The washed culture was then

boiled at 95°C in a heat block for 10 minutes to heat kill the bacteria. The resulting suspension was centrifuged at maximum speed for 1 minute and the supernatant subsequently removed; this could then be employed in embryo microinjection.

Table 2.6: Bacterial strain growth conditions

Bacterial strain	Growth temperature (°C)	Antibiotic	Concentration (μg/mL)
Erwinia carotovora carotovora 15 (Ecc15)	30.0	Rifampicin	25.0
BL 21 RFP-Escherichia coli	37.0	Ampicillin	100
DH5α Escherichia coli	37.0	None	-
Mg1655 Escherichia coli	37.0	None	-
Micrococcus luteus	37.0	None	-
TTO1 Photorhabdus luminescens	28.0	Rifampicin	25.0
4134 Salmonella typhimurium	37.0	-	-

Not all cultures required antibiotic selection, hence those that were grown solely using LB medium are indicated with '-'.

Table 2.7: Final OD values of Bacterial Stains

Bacterial strain	OD_{600}	Assay
Erwinia carotovora carotovora 15	0.1	2.9B, 2.9E
	0.5	2.9C
	1	2.10B, 2.10C, 2.14, 2.15C
BL21 RFP-Escherichia coli	10	2.9D
DH5a Escherichia coli	1	2.9A
Mg1655 Escherichia coli	1	2.9B
Micrococcus luteus	1	2.9B
TTO1 Photorhabdus luminescens	1	2.9A
4134 Salmonella typhimurium	1	2.9A

Further details of assay protocols can be found in the corresponding sections as indicated by the assay number.

2.7 Microinjection of *Drosophila* embryos

Embryos were mounted as described in 2.5B and subsequently injected using the Eppendorf FemtoJet microinjector platform. An Eppendorf needle was loaded with 5μL of the appropriate culture or control sterile endotoxin-free PBS and the tip broken on the edge of the coverslip containing the embryos to allow consistent flow of the culture. Embryos subsequently received an injection in the anterior region of approximately 3nL of culture or control PBS for 1 second at an injection pressure (P_i) of 50-100hPa and compensation pressure (P_c) of 20hPa. Post injection, embryos were incubated at 22°C in a box containing moistened tissue for the appropriate time period.

2.8 Sterile laser ablation of *Drosophila* embryos

To generate RNA samples of wounded embryos, *Drosophila* embryos were prepared and Stage 15 selected on agar plates as detailed above. All embryos were then transferred to a drop of Halocarbon 700 oil on a Petri dish. Embryos were then individually transferred using a tungsten needle to PetriPerm hydrophobic membranes, flanked by two 18x18cm glass coverslips, and orientated ventral side up. Embryos were arranged in a grid of 3 x 10, so that 30 embryos were contained upon each membrane. The embryos were covered with extra Halocarbon 700 oil using a Tungsten needle, to ensure embryos were kept hydrated. A 24x32mm glass coverslip was then placed on top of the flanking coverslips using tweezers so that the embryos were effectively covered. Magic tape was then used to secure the coverslip to the membrane. Wounding was performed using a Spectra Physics Nitrogen laser attached to a Leica SP5 confocal microscope. Embryos were wounded once in the centre of the ventral side. The embryos were then incubated at 25°C for 2 hours before the coverslip was removed from the membrane. The embryos were then transferred to an inverted 40μm cell strainer inside a Petri dish and washed once with 100% heptane to remove the Halocarbon 700 oil. Once satisfied that the oil had been removed, the embryos were transferred into a

sterile microcentrifuge tube and 250μL Qiazol added. The samples were then frozen at -80°C until required.

2.9 Infection assays

A. Bacterial Infection of *Drosophila* embryos to determine the time-course of the immune response

To assess if embryos were able to mount an effective immune response to a bacterial stimulus, cohorts of 25 embryos containing the *Drosocin-GFP* (*Drc-GFP*) transgene were selected and processed, before being mounted for microinjection as described in Section 2.7. Embryos were microinjected either with live or heat killed *E. coli* (OD_{600}=10) or *Ecc15* (OD_{600}=1) as described in 2.7. Embryos were incubated on their injection apparatus in a box containing moist tissue at 22°C for 6 hours post injection, at which point GFP expression and localisation was visualized using a Leica M716F fluorescence dissecting scope, Leica DC350FX camera and Adobe Photoshop C53 software linked to a TWAIN module for Leica DC cameras.

B. Mortality assay

To assess embryo viability post injection, embryos were employed in a mortality assay. Embryos were selected and mounted for injection as described in 2.5B and subsequently injected with either sterile endotoxin-free PBS or bacterial suspension as detailed in Section 2.7. ≥100 embryos were injected per experimental replicate (n=3). Non-treated control replicates were generated by selecting groups of 100 embryos on apple juice agar plates, transferring them to clean agar plates using a tungsten needle and arranging them in an array of 10x10 lines. Sufficient Halocarbon 700 oil was then used to cover them to prevent desiccation. All samples were then stored in parafilm-sealed humidified boxes at 25°C for the appropriate time period; 24 hours for embryos injected at Stage 15 and 30 hours for embryos injected at Stage 11. At this time point, mortality was measured by counting the numbers of dead

and live larvae and embryos. The total percentage survival was calculated for each replicate and a mean value for each treatment and experiment derived.

C. 20-hydroxyecdysone rescue assay

To assess if 20-hydroxyecdysone (20-HE) could rescue the immune response and survival of Stage 11 embryos injected with bacterial species, a rescue assay was performed. *Drc-GFP* Stage 15 and Stage 11 embryos were selected and mounted for microinjection as described in 2.5B. Groups of ≥100 Stage 15 and Stage 11 embryos were subsequently injected as described in 2.7 with either sterile endotoxin-free PBS, 25μM 20-HE, *Ecc15* (OD=0.5) or *Ecc15* (OD=0.5) + 25μM 20-HE. The coverslips on which the embryos were adhered were removed from their respective slides and transferred to apple juice agar plates. Embryos were then incubated at 25°C post infection in a humidified container. The time-course, localisation, intensity and numbers of *Drc-GFP* expression within embryos were monitored for 30h post injection, as well as the developmental stage of the individual embryo using yolk sac morphology. Images of embryos were obtained at 12 hours post infection using a Leica M716F fluorescence dissecting scope, Leica DC350FX camera and Adobe Photoshop C53 software linked to a TWAIN module for Leica DC cameras.

D. Phagocytosis assay

To determine if bacteria were effectively phagocytosed, embryos containing GFP-expressing plasmatocytes were employed in a phagocytosis assay. Embryos were dechorionated and maintained in distilled water described above. The 70μm cell strainer was subsequently removed from the water and dried. Stage 15 embryos were selected directly from the basket using a tungsten needle and transferred to double sided tape adhered to lateral edge of a 24x32mm coverslip. The embryos were orientated for microinjection in accordance with Section 2.5B. The coverslip was then adhered to a glass slide using a drop of Halocarbon-700 oil. Embryos were desiccated for 8 minutes in a box of silica salt before transference to a box containing moist tissue.

Live *RFP-E. coli* were cultured and prepared as described in Section 2.6. The OD_{600} value was adjusted to a value of 1 using sterile endotoxin-free PBS. Embryos were injected as described in 2.7. Post-injection, two 22x22mm were adhered in a stack using nail varnish. These were then attached to the slide using double-sided tape adjacent to the embryos. A further 22x22mm coverslip was attached to the remainder of the embryo coverslip using double sided tape. A 24x32mm coverslip was then carefully lowered over the embryos to rest on these newly applied supports and secured in place with nail varnish. Embryos were then subjected to time-lapse confocal imaging, with the parameters defined in Section 2.14.

E. Determination of Colony Forming Units Post Infection

To determine how many Colony Forming Units (CFUs) of bacteria were present in embryos 7.5 hours after infection, embryos were microinjected as described in 2.7 and incubated at 25°C for 7.5 hours on their injection apparatus in a humidified box to allow time for gene transcription to occur. After this time period, embryos were removed from the injection apparatus by washing slides with 100% heptane using a Pasteur pipette. Embryos were washed into a sterile 50mL Falcon tube and excess heptane removed. Using a Pasteur pipette, embryos were transferred to a sterile microcentrifuge tube and washed once with 1mL of sterile PBS to remove any remaining heptane. Embryos were subsequently crushed in 300µL sterile PBS using a sterile plastic pestle until no particulate remained. The suspension was spread on to LB agar plates in triplicate, with 100µL inoculated on to each plate. LB plates were incubated at the appropriate temperature overnight to allow for bacterial growth and the number of CFUs present after 18 hours recorded.

2.10 Antibody staining

A. Staining of non-treated embryos

Stage 15 embryos were dechorionated as described previously and transferred to a 1:1 mix of 100% heptane and 4% formaldehyde. Embryos were then fixed for 20 minutes on a roller before removal of the formaldehyde phase. 500μL of 100% methanol was added to remove the embryonic vitelline membrane. Subsequently, devitellinised embryos were transferred to a clean microcentrifuge tube using a Pasteur pipette and washed three times with 500μL 100% methanol. Embryos were permeabilised three times with washes of 500μL 0.1% Triton-X in PBS (PBT) and subsequently washed three times in 500μL 0.1% Triton X, 1% BSA in PBS (PATx) for 20 minutes on a roller, to block non-specific staining. Embryos were transferred to a clean microcentrifuge tube using a Pasteur pipette and excess PATx removed. Primary antibodies diluted to the required concentration in PATx, as detailed in Table 2.6, were added and the samples incubated overnight on a roller at 4°C. Samples were washed three times with PATx for 20 minutes on a roller before application of the secondary antibody, diluted as displayed in Table 2.7. 1μL FITC-phalloidin was added to stain F-actin as required. Samples were incubated with secondary antibody for 2 hours at room temperature on a roller before washing three times with PATx as above. 200μL of 1,4-diazabiycylo[2.2.2]octane (DABCO) was subsequently applied to the samples and enabled them to be stored at 4°C until required.

B. Staining of treated embryos

Embryos were injected either with sterile endotoxin-free PBS or *Ecc15* and incubated at 25°C for 6 hours to allow expression of transgenic constructs. After this period, embryos were removed from their injection apparatus as described above and collected into a 50mL Falcon tube, from where they were transferred using a Pasteur pipette into 1mL of 100% heptane in a glass vial. 1mL of 4% formaldehyde was added to the vial and the embryos fixed at the interphase on a shaker for 20 minutes at room temperature. The embryos

were subsequently transferred to an inverted 70μm cell strainer using a Pasteur pipette and any excess heptane removed. The embryos were directly transferred to double-sided tape and the tape subsequently adhered to a Petri dish. PBS was used to cover the embryos to ensure that they remain hydrated. Once adhered to the tape, embryos were hand devitilinised using tweezers and collected into a microcentrifuge tube using a Pasteur pipette. Excess PBS was removed and the embryos were employed in consecutive washes of 25%, 50% 75% and 100% methanol in PBS. Immunostaining was then carried out as described above.

2.11 Primer design, synthesis and validation

A. Primer design

Gene sequences were downloaded from National Center for Biotechnology Information (NCBI) website (accessed from http://www.ncbi.nlm.nih.gov/) Primer pairs were then designed using the online freeware Primer3 (accessed from http://frodo.wi.mit.edu/primer3/) using the table of themodynamic parameters from Breslauer *et al.* (1986) and the search parameters outlined in Table 2.8.

Table 2.8: Parameters used in Primer Pair Generation

Parameter description	Parameter value
Product size range	80-120bp
Number to return	20
Minimum primer T_m	59.0°C
Optimum primer T_m	60.0°C
Maximum primer T_m	61.0°C
Maximum T_m Difference	1.0°C
Maximum primer GC%	70.0%
Maximum self complementarity	2-4bp
Maximum 3' Self complementarity	0-3bp

Parameters for primer design accounted for a range of primer features, including percentage GC content (%GC), annealing temperatures of primer pairs (Tm) and pair complementarity.

Upon successfully generating primers with acceptable thermodynamic properties, the structure of the gene region at 60°C was predicted using Mfold freeware (accessed from http://mfold.rna.albany.edu/?q=mfold) using the DNA structure application. It was assessed whether the primers would bind to linear regions or hairpin loops within the gene structure. If it was the case that the primers would bind within hairpin loops, those whose 3' end extended up a hairpin loop structure (Figure 2.3B) were rejected, as this has been shown to significantly decrease the efficiency of a PCR reaction (Singh *et al.,* 2000). Those whose 3' ends extended down hairpin loops (Figure 2.3A) were accepted for the next phase of validation.

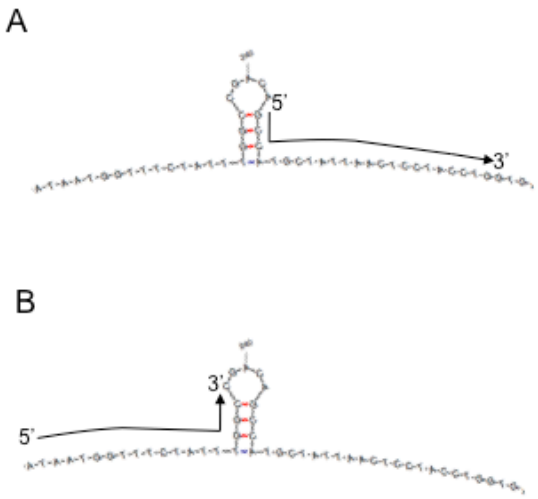

Figure 2.3: Primer binding within predicted hairpin loops in target genes. Primers annealing to target sequence down a hairpin loop 5'-3' (A) were progressed through the design process, whereas those which extended up a loop 5'-3' were discarded.

In the final stage of the primer design protocol, the primer sequences were entered into the nucleotide Basic Local Alignment Search Tool (BLASTn) hosted on the NCBI website (accessed from http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) to determine *in silico* the range of genes that the primers would theoretically identify in PCR reactions, and hence if the primers would bind specifically to the gene of interest and if any off-target sequences would also be selected. To

do this, the nucleotide collection was screened to identify highly similar sequences. If both forward and reverse primers selected the gene of interest with high identity and a low expect (E) value, in comparison to other potential hits, then the primers sequences were sent for synthesis. The primers generated are listed in Table 2.9; further details on themodynamic properties of specific primers, the sites of primer binding relative to secondary structures and *in silico* assessment of primer specificity can be viewed in Appendices 3, 4 and 5 respectively. Primer sequences for internal control and AMP genes were provided by B. Lemaitre, EPFL, Lausanne, Switzerland, and are shown in Table 2.10. Primer sequences for evaluating ecdysone-related genes, accessed from Hackney et al. (2012), are displayed in Table 2.11.

B. Synthesis and Reconstitution

Primer sets were synthesized by Invitrogen (Paisley, UK) to a 25nmol scale. Upon receipt, primers were reconstituted in DEPC-treated water to a 100μM concentration. A 10μM working stock in DEPC-treated water was then generated for use in quantitative PCR (qPCR) validations and further experiments.

C. Genomic DNA (gDNA) isolation

5 male and 5 female flies were selected to procure approximately 25mg of tissue. The Dneasy Blood and Tissue Kit was used to extract the total DNA from these flies, using the spin column protocol. The flies were homogenized using a sterile plastic pestle in 180μL Buffer ATL to release their DNA. 20μL Proteinase K was then added and the whole suspension mixed vigorously by vortexing. The suspension was then incubated at 56°C for 3 hours, vortexing occasionally, until the tissue was maximally lysed. After this period of time, the sample was vortexed for 15s and 200μL Buffer AL was added and vortexed again. 200μL 100% ethanol was introduced to the sample and followed by thorough vortexing. The mixture was then transferred into a DNeasy Mini spin column placed inside a 2mL collection tube, where the DNA would bind to the column membrane. The column was centrifuged at 6000 x g

Table 2.9: Immune Primer Sequences generated

Gene target	Forward primer (5'-3')	Reverse primer (5'-3')
Dopa decarboxylase (Ddc)	TGCTGGTGAACTTTGACT	AAGAGGGTCCACATTGAA
Growth Arrest and DNA Damage-inducible 45 (GADD45)	GCCATCAACGTGCTCTCC	AGATGTCGTTCTCGTAGCAAAG
Masquerade (mas)	GAGGGGAGAACTCTGGTGTG	GCAGCTTCTCGGTAATCCAA

Table 2.10: AMP Gene and Internal Control Gene Primer Sequences contributed by B. Lemaitre

Gene target	Forward primer (5'-3')	Reverse primer (5'-3')
Attacin	CCCGGAGTGAAGGATG	GTTGCTGTGCGTCAAG
Cecropin	GAACTTCTACAACATCTTCGT	TCCCAGTCCCTGGATT
Defensin	GTTCTTCGTTCTCGTGG	CTTTGAACCCCTTGGC
Diptericin	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG
Drosocin	CCATCGTTTTCCTGCT	CTTGAGTCAGGTGATCC
Drosomycin	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT
Metchnikowin	AACTTAATCTTGGAGCGA	CGGTCTTGGTTGGTTAG
rp49	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG

Table 2.11: Ecdysone-related Gene Primer Sequences

Gene target	Forward primer (5'-3')	Reverse primer (5'-3')
Defective in avoidance of repellents (Dare)	ATCTAGTTGCGTGGATACGGGCAT	AGCCAGCCAGCTACATAAAGTCCA
Ecdysoneless (Ecd)	AGCGACTCGGATGAGTGGTTGAAT	GGCATTCATTTGTCCGTTCGGCTT
Ecdysone inducible protein (Eip) 71CD	ACGGAGGTGCTGGAAATCGACTAT	TGGTCAGGCCATACTCATGGTTGT
Eip74EF	TTTCATCAAGTGGACGAACCGGGA	CATGTCCGGCTTGTTCTTGTGCAT
Eip78C	ATGTAAGCGGCGTACGTGTGAAGA	TTATTGGCACTATTGCAACCGCCC
Ecdysone oxidase (Eo)	AGACCTACTCTCGCCTGCAACAA	TGTTTCATCCGTGGTACACCCAGT
Molting defective (Mld)	ACTGTGCGAACGGAATTGAACAGC	TGAGGATGCCATTGAGTGTGGTCT
Neverland (Nvd)	AGCAACTTGTGTGTCATGCTTGGG	TTGGCTCCTAGGTGAGGGCAATAA
Phantom (Phm)	TGGGAAACCAAGAAGCTGAC	CGATTTCCTCCTGCTCTCAC
Prothoracicotropic hormone (Ptth)	TGGTGACCACCAAACGCAATGATG	ATCAGAAAGCGAGGGAAGTGCAGA
Ultraspiricle (Usp)	CCTGTGCCAAGTGGTCAACAAACA	ATCCAAGCGGCTTTCAGCAGAATC

for 1 minute and the flow through discarded. The column was transferred to a new collection tube and 500μL Buffer AW1 added, before centrifuging and discarding the flow through as before. 500μL Buffer AW2 was then added and the column centrifuged for 3 minutes at 20000 x g and the flow through discarded, to dry the membrane. The column was finally placed into a 1.5mL microcentrifuge tube. 100μL Buffer AE was transferred directly onto the membrane. The column was incubated at room temperature for 1 minute before centrifuging at 6000 x g for 1 minute to elute the purified DNA into the microcentrifuge tube. The same process was then repeated using a further 100μL of Buffer AE to procure maximum DNA yield. The yield of DNA was subsequently quantified using an Eppendorf Biophotometer. A 1:50 dilution of DNA was performed in an UVette cuvette using DEPC-treated water. DEPC-treated water was used to calibrate the biophotometer and the OD at 260, 230 and 280nm measured. The resulting purified DNA sample was then stored at -80°C until required.

D. Quantitative PCR (qPCR) Validation and analysis

For primers that were to be employed in qPCR, it was necessary to validate their performance and percentage binding efficiency within this assay. This included those whose sequences were supplied by B. Lemaitre and from Hackney *et al.* (2012). The gDNA procured in 2.11C was used as the template for these reactions. 1:10 and 1:100 dilutions of the gDNA in DEPC-treated water were generated, so as to generate a standard curve of gDNA amplification. A qPCR reaction mix was generated to test the binding efficiency of each primer for each dilution of gDNA. This consisted of 7.5μL qPCR supermix, 0.75μL of 10μM stocks of each respective forward and reverse primer and 3.5μL of DEPC-treated water for each reaction required. 12.5μL of each mix was transferred to its corresponding well in a 96-well qPCR plate (Figure 2.4). 2.5μL of either neat, 1:10 or 1:100 diluted gDNA was then added to the wells A negative control was also included, whereby 2.5μL of DEPC-treated water was added to the corresponding reaction mix in place of gDNA. Each reaction was replicated in duplicate for each gDNA dilution to

ensure accuracy. Finally, the plate was sealed with optical film and centrifuged for 5 minutes at 106 x g and 4°C before being transferred into the Biorad StepOne qPCR system. Amplification of the gDNA was performed at 40 cycles, with an initial denaturation phase of 95°C for 2 minutes and then cycles of 95°C for 10 seconds and 60°C for 1 minute. Amplification plots were generated to study product amplification. Standard curves of threshold cycle (C_t) against concentration of gDNA for each primer pair were generated using the StepOne software (v2.2.2). Primer efficiency was calculated using Equation 1:

Efficiency (E) = $10^{(-1/\text{gradient of standard curve})}$ **Equation 1**

Melt curve analysis to assess the specificity of the qPCR product was achieved by melting the product at 95°C then reducing the temperature to 55°C and subsequently increasing the temperature by 0.5°C increments to 95°C.

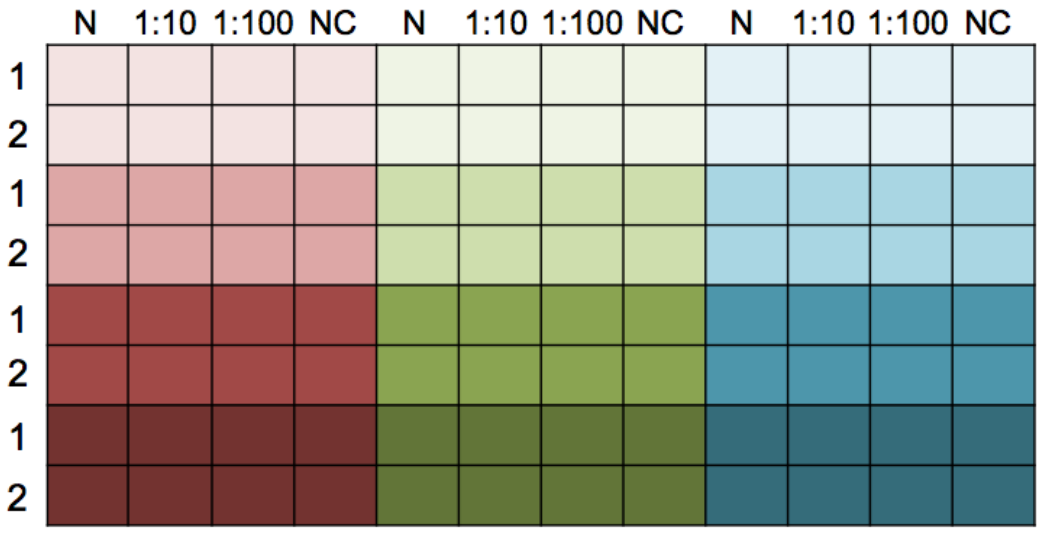

Figure 2.4: Layout of a qPCR plate for primer validation. Coloured squares indicate the wells of the plate that contain the primers targeting different genes, or alternate primer sequences for the same gene. Each row within a coloured block represents one replicate, either 1 or 2. To generate a standard curve to measure efficacy of primer binding, reactions containing either a neat (N), 1:10 or 1:100 dilution of gDNA were established. A negative control (NC) well was also included for each replicate to assess primer dimer formation.

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2.12 Bacterial infection of *Drosophila* embryos for RT-qPCR analysis

A. Microinjection and post-injection processing

Embryos of the desired developmental stage were selected and mounted for microinjection as described in 2.5B. Groups of 200 embryos were injected with either sterile endotoxin-free PBS, *Mg1655 E. coli*, *M. luteus* or *Ecc15* as required, in accordance with 2.7. Post-injection, embryos were incubated as described in 2.9E. At 2 hours post infection, embryos were removed from their injection apparatus, by washing into a 70μm cell strainer using 100% heptane. Embryos were subsequently recovered into a sterile microcentrifuge tube containing 100μL 100% heptane using a paintbrush. Excess heptane was then removed and 250μL Qiazol added. Samples were then snap frozen in dry ice and stored at -80°C for at least 24 hours or until required.

B. RNA extraction

Samples were defrosted on ice and processed using the RNeasy Mini Kit in combination with Qiazol. Embryos were homogenised in the 250μL Qiazol in which they were frozen, using a sterile plastic pestle, until no particulate remained. The volumes of sample were then increased to 1mL with further Qiazol and the samples left to incubate at room temperature for 5 minutes. 200μL 100% chloroform was added to each sample and the mixtures vigorously mixed for 15 seconds. Subsequently incubating the mixtures at room temperature for 10 minutes allowed for initial phase separation. This was followed by centrifugation at 4°C and 15,200 x g for 15 minutes to further aid phase separation. The aqueous phase from each sample was transferred into individual tubes containing 600μL 70% ethanol and gently mixed. The samples were then transferred quickly into individual spin columns inserted into 2mL collection tubes. The columns were centrifuged for 20 seconds at 9,300 x g at room temperature. The flow through was discarded and the column replaced in the collection tube. 500μL Buffer RPE was added to each column and the samples were centrifuged as before for 20 seconds. The flow through was discarded and this step repeated to was the column membrane. Centrifugation

was carried out as before for 2 minutes. The spin column was then placed into a clean 2mL tube and centrifuged at full speed for 1 minute at room temperature to eliminate any possible carry over of Buffer RPE. The spin column was finally placed in a 1.5mL tube and 50µL DEPC-treated water was pipetted directly onto the membrane. The samples were then eluted by centrifugation at 9,300 x g for 1 minute at room temperature into a clean microcentrifuge tube. The resulting RNA was either frozen at -80°C until required or applied directly to DNase treatment.

C. TURBO DNase Treatment

DNase treatment of RNA samples was undertaken to remove any genomic DNA present. 5µL 10X TURBO DNase buffer and 2µL TURBO DNase was added to all RNA samples and the contents mixed gently. The samples were incubated at 37°C in a heat block for 1 hour to allow digestion to occur. Following this period, 10µL DNase Inactivation Reagent was added to the samples and the suspension mixed well. The samples were subsequently incubated at 25°C in a heat block for 5 minutes, during which time they were mixed twice, before being centrifuged at 1,000 x g for 1.5 minutes to separate the inactivation reagent from the RNA sample itself. The RNA was then transferred to a clean tube and the concentration subsequently determined.

D. RNA quantification

RNA quantification was subsequently performed using the Qubit Quantification Platform and Quant-iT assay kit. A working solution of the Quant-iT RNA probe was generated by diluting 1:200 in Quant-iT RNA buffer, ensuring 1µL of probe for each RNA sample and all RNA standards to be quantified. 199µL of working solution was added to 1µL of RNA sample in their respective Quant-iT assay tubes. 190µL of working solution was added to 10µL of the RNA standard solutions (0 and 10ng/µL RNA concentrations) required for platform calibration in Quant-iT assay tubes. All tubes were then vortexed for 2-3 seconds and incubated at room temperature for 5 minutes. The Qubit Quantification platform was calibrated using the standards and the samples

subsequently measured. The dilution factor of the assay samples was then extrapolated to determine the RNA concentration of the original samples. Subject to a satisfactory RNA concentration, the samples were then employed in RT-qPCR.

E. RT-qPCR

Upon satisfying RNA validation constraints, the RNA samples were employed in RT reactions to generate cDNA using the Superscript III First Strand Synthesis System. The amount of RNA per RT reaction was standardized to 1μg. To this volume of RNA, 1μL of random Oligo$(dt)_{20}$ primers and 1μL 10mM dNTPs were added and the volume made up to 10μL with DEPC-treated water in a sterile PCR tube. The mixes were then incubated at 65°C for 5 minutes in a PCR block to allow for random primer binding to the RNA and to remove any secondary structure before chilling on ice for at least 1 minute. An RT mix was generated, consisting of 2μL 10X Buffer, 4μL 25mM $MgCl_2$, 2μL 0.1M DTT, 1μL RNaseOUT RNase Inhibitor and 1μL Superscript III reverse transcriptase per reaction; 10μL was dispensed into each PCR tube. The resulting mixes were then incubated in a PCR block at 50°C for 50 minutes and subsequently 85°C for 5 minutes to inactivate the reverse transcriptase. 1μL RNase H was added to each reaction and incubated at 37°C for 20 minutes to degrade any remaining RNA. Finally, 180μL DEPC-treated water was then added to give a final cDNA concentration of 5ng/μL. The RT reactions were stored on ice, ready to be used in qPCR, or frozen at -80°C until required.

qPCR was carried out using the StepOne Real-Time PCR Detection System. 5μL of cDNA (≈25ng) was transferred to each individual well of a 96-well qPCR plate using an Eppendorf Research Pro repeat pipettor. A master qPCR reaction mix was generated. This consisted of 7.5μL qPCR supermix, 0.75μL of 10μM stocks of each respective forward and reverse primer and 3.5μL of DEPC-treated water for each reaction required. 12.5μL of each mix was transferred to its corresponding well in a 96-well qPCR plate. Plates were sealed using optical film and centrifuged for 5 minutes at 4°C at 106 x g.

Amplification of the cDNA was performed for 40 cycles, with an initial denaturation phase of 95°C for 2 minutes and then cycles of 95°C for 10 seconds and 60°C for 1 minute. Amplification plots were generated to study product amplification.

2.13 Microarray Studies

A. Total embryo RNA isolation and quality control

Groups of 200 Stage 15 *w; srp-Gal4, UAS-GFP; crq-Gal4, UAS-GFP* embryos were mounted and microinjected as detailed in 2.5B, with either sterile endotoxin-free PBS, *M. luteus* (OD=1) or *Ecc15* (OD=1). Control, non-infected embryo groups were also established by collecting 200 Stage 15 embryos and transferring them to a drop of Halocarbon-700 oil on a slide. Each treatment was replicated in triplicate. All injected and control samples were incubated at 25°C for 2 hours to allow for gene transcription to occur. After this time period, the embryos were harvested from the injection or incubation apparatus. Non-infected embryos were transferred to an inverted 70μm cell strainer using a tungsten needle and 100μL 100% heptane added to them in a drop-wise fashion to remove the Halocarbon 700 oil. Embryos were transferred to 100μL 100% heptane in a RNase-free microcentrifuge tube using a paintbrush, to ensure that all oil was removed. Excess 100% heptane was removed and 250μL Qiazol added in a fume hood. Samples were then snap frozen in dry ice and stored at -80°C until required.

Total RNA was isolated from the samples using the RNeasy Mini Kit as described in 2.12B. The quantity and quality of the resulting RNA was examined using the Experion Automated Electrophoresis System in conjunction with the Experion RNA StdSens Analysis Kit. Before a run, the system electrodes were cleaned by inserting a cleaning chip filled with 800μL cleaner for 2 minutes before rinsing for 5 minutes and then 60 seconds via a cleaning chip filled with 800μL DEPC-treated water. The kit reagents were

equilibrated at room temperature for 20 minutes prior to use. After this period, 600μL RNA gel was transferred into a spin filter tube and centrifuged at 1,500 x g for 10 minutes. 65μL of gel was then transferred into a microcentrifuge tube. 1μL stain was added to the tube and the contents vortexed and subsequently foil-wrapped to protect from light. 2μL aliquots of each sample and the RNA ladder were transferred to individual microcentrifuge tubes and denatured at 70°C for 2 minutes in a heat block before cooling immediately on ice for 5 minutes. A chip was primed by pipetting 9μL of filtered gel-stain solution into the highlighted gel-strain (GS) well (Figure 2.5). The chip was then placed in the priming station and pressure setting B and time setting 1 used to prime the chip. 9μL of gel-strain solution was pipetted into the other GS well and 9μL filtered gel was loaded into the gel (G) well. 5μL of loading buffer was transferred into each sample well (1-12, Figure 2.5) as well as the ladder (L) well. 1μL of denatured ladder was pipetted into the L well and 1μL of each RNA sample into individual sample wells. The chip was then vortexed in the vortex station for 1 minute. The chip was then placed within the Experion Automated Electrophoresis System and the run started. Progress was monitored using the Experion software (V3.20).

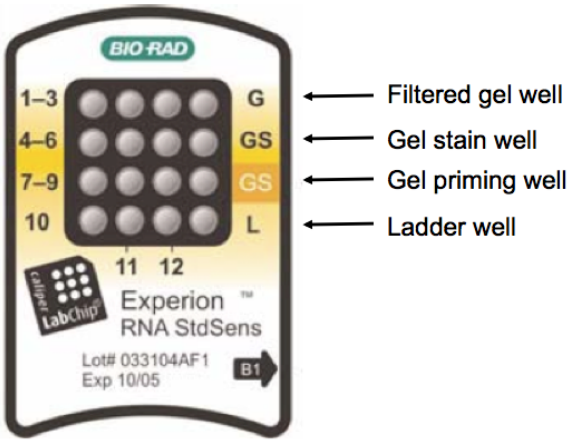


Figure 2.5 Schematic to show the layout of Experion Chip (Biorad Laboratories, 2010). Gel-stain solution was loaded into the highlighted gel priming (GS) well to prime the chip. Further gel-stain was then added via the GS well, and filtered gel added in the well labeled G. Denatured RNA ladder was loaded via the specified ladder (L) well, and denatured RNA samples loaded in the wells annotated 1-12.

B. Sample preparation for hemocyte microarray studies

Hemocyte samples were initially prepared in a similar manner to preparation of total RNA samples. A minimum of 1000 Stage 15 *w; srpGal4, UASGFP; crqGAl4, UASGFP* embryos were injected with either sterile endotoxin-free PBS or *Ecc15* as described above before being incubated at 25°C in a portable incubator for two hours. Upon completion of this incubation, the embryos were washed with 100% heptane to remove halocarbon oil and embryo glue and were collected into a 70μm nylon cell strainer. Embryos were then transferred in groups of 400 into 900μL Schneiders media in a sterilized borosilicate tissue grinder. The embryos were homogenized by four strokes of the pestle. The resulting cell suspension was titurated two times and then transferred into a sterile microcentrifuge tube. 50μL 2.5% trypsin was added to further digest embryo tissue and the suspension mixed. The cell suspension was subsequently centrifuged at 800rpm at 4°C for 5 minutes to remove debris. The supernatant was removed and passed through a 40μm nylon cell strainer into a Petri dish on ice. This was then transferred to a clean microcentrifuge tube and centrifuged at 2000rpm for 10 minutes at 4°C. The supernatant was discarded and 200μL Seecoff buffer added. The resulting cell suspension was then used to determine the yield of hemocytes from this process or for creating hemocyte samples for microarray analysis using FACS.

C. Fluorescence Assisted Cell Sorting (FACS) of hemocytes

Hemocytes samples were collected by FACS within the Flow Cytometry Suite of the School of Medical and Vetinary Sciences, University of Bristol, by Dr. A. Herman. Cells were counted using a BD Influx Cell Sorter. Measurement of specific parameters permitted the development of a FACS protocol that could be employed in future sorting experiments, to provide consistent sorting between replicates (Figure 2.6). Forward scatter counting (FSC) was utilized to count and estimate the size of cells passing through the detector. Side scatter counting (SSC) was employed to measure cell complexity and granularity. Gating was applied to the population of cells displaying appropriate size and complexity. Measurement of trigger pulse width within the gated cell population permitted for the further selection of cells that were

observed as singlets. Of the singlet cell population, those that displayed GFP-associated fluorescence, termed GFP-positive (GFP+) cells, were identified. Gating was established to count and sort only GFP+ cells that emitted the highest levels of GFP-associated fluorescence upon stimulation. As hemocytes were the only cell type within the embryos to express GFP, this protocol could be employed to effectively isolate hemocytes from a mixed population of cells. GFP+ cells were sorted into 200μL RNALater and stored at 4°C until required. For initial cell characterization experiments, GFP- cells were also separately sorted into 200μL RNALater.

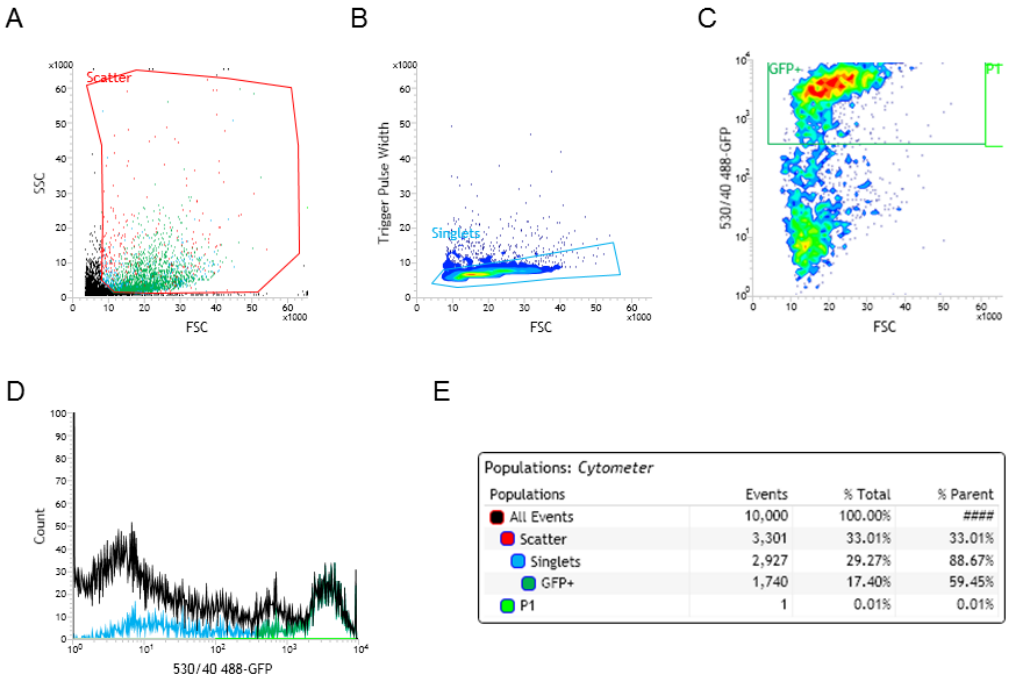


Figure 2.6: Hemocyte FACS protocol, optimized by Dr. A. Herman. A FACS protocol was optimized to efficiently sort GFP-positive (GFP+) hemocytes from a mixed cell suspension. Cells of the correct size and complexity were initially gated (A) and from this selected population, single cells were identified and a second gate established (B). From the single cell population, GFP+ cells were identified and a final gate established (C). The numbers of GFP+ cells as a percentage of the total cell population could then be established (D and E).

D. Hemocyte RNA extraction

Upon attaining a minimum of 50,000 sorted GFP+ cells for each treatment, the samples for each treatment were pooled where necessary and RNA isolated from the hemocytes using the RNAqueous Micro Kit. The cell suspensions were vortexed vigorously in 100μL Lysis solution. 50μL 100% ethanol was

added to each before vortexing briefly. The mixtures were then loaded into their respective Micro Filter Cartridge Assembly and centrifuged for 10 seconds at maximum speed. 180μL of Wash Solution 1 was added and centrifugation repeated. Two washes with 180μL of Wash Solution 2/3 were performed for each column and centrifugation repeated. The flow through was discarded and the columns centrifuged at maximum speed for 1 minute to remove any residual fluid and to dry the filter. The columns were then transferred to microcentrifuge tubes. 10μL of Elution Solution, preheated to 75°C, was applied to the centre of each filter and the columns stored for 1 minute at room temperature. The assembly was then centrifuged at maximum speed for 30 seconds to elute the RNA from the filter. This elution step was then repeated with a further 10μL Elution solution to maximize RNA yield.

DNase I treatment was then employed to ensure all traces of genomic DNA were removed from the resulting RNA samples. 2μL 10X DNase I buffer and 1μL DNase I was added to each sample. The reaction was incubated at 37°C for 20 minutes. 2μL DNase Inactivation Reagent was subsequently added to each reaction. The reactions were then centrifuged at maximum speed for 1.5 minutes to pellet the Inactivation Reagent. RNA was transferred to a fresh microcentrifuge tube and stored at -80°C until required. The quality of the resulting RNA was examined using the Experion Automated Electrophoresis System in conjunction with the Experion RNA StdSens Analysis Kit. Quantification of hemocyte RNA was determined using the Qubit Quantification Platform and Quant-iT assay kit.

E. Microarray protocol

All microarray experiments were performed by Dr. B. Fischer at the Cambridge Systems Biology Centre, University of Cambridge. All samples were amplified using the Switch Mechanism at the 5’ end of Reverse Transcript (SMART) amplification method. For the total RNA samples, 100ng of RNA was amplified using 18 cycles. For the hemocyte samples, the starting quantity of RNA was 50ng and thus 24 cycles of amplification was required. The samples were then

subjected to quality control protocols to ensure that their quality was sufficient for microarray experiments. This included checking for the presence of genomic DNA within samples and measuring the optical density of the samples at 260, 230 and 280 nm. Upon satisfactory quality control, the samples were hybridized to FlyChip arrays. Samples were assigned in a random fashion to the Cy3 or Cy5 channels, and this dye swap taken into account upon data analysis.

2.14 Image acquisition

To obtain 5X images of whole *Drc-GFP* and *Drs-GFP* embryos and flies, a Leica DC350FX camera attached to the Leica M716F fluorescence dissecting scope, and Adobe Photoshop C53 software linked to a TWAIN module for Leica DC cameras were employed. A zoom of 4.0 was used for images of both adult flies and embryos. For imaging of immunostained embryos to visualize the localisation of *Drc-GFP* expression and the localization of the ecdysone receptor (EcR) in discrete tissues, as well as the presence of PGRP-LC-GFP in live embryos, a spinning disk microscope was employed using Volocity Image Analysis software (v.6.3).

To obtain z-stack images of embryos employed in the phagocytosis assay, imaging was performed on the aforementioned Zeiss-LSM510 confocal laser scanning microscope, under 63X objective. Z-stacks with 1μm spacing were employed to capture images of labeled hemocytes and bacteria. The resulting z-stacks were used to calculate the percentage of phagocytosed bacteria per embryo.

2.15 Data processing and Statistical analyses

A. Image data processing

Image data was processed using the software ImageJ (v1.42q). For phagocytosis assay data, orthological projections were employed to determine if bacteria had been effectively phagocytosed. The mean values of bacteria phagocytosed as a percentage of total bacteria injected were subsequently calculated for each comparison group. Orthological projections were also employed to assess the localization of *Drc-GFP* expression with embryos.

B. RT-qPCR data processing

To process RT-qPCR data pertaining to AMP gene expression, the threshold cycle (C_t) for each reaction was recorded and the relative amounts of cDNA present for each gene were then calculated using the equation:

$$N_A/N_B = 2^{-\Delta Ct} \qquad \text{(Equation 2)}$$

Where C_t is the threshold cycle and NA and NB represent the relative level of gene expression. Expression levels for each target gene were calculated with reference to the internal control gene, *rp49*, and subsequently normalized according to control levels and the fold difference in expression and standard deviation subsequently calculated.

RT-qPCR data pertaining to the relative expression of 20-HE signaling components was processed entering the recorded C_t values for each reaction into the Relative Expression Software Tool 2009 software (v1.0; Qiagen, UK). Analysis of relative gene expression was normalised with reference to *rp49* expression, to compensate for variation.

C. FACS data processing

Dot plots of FSC and SSC on a linear scale were used to determine cells of the appropriate size and complexity, and permitted for initial gating. Heat map plots of FSC against trigger pulse width on a linear scale were used to identify

and effectively gate singlets within the resulting population. Heat map plots of FSC against levels of GFP-associated fluorescence emission (488-GFP) using a log scale were utilized to identify and gate the cells expressing the most intense levels of GFP-associated signal from the single cell population. Histograms were subsequently used to view the GFP+ cell population as a fraction of the total cell population and a cytometer function used to calculate the percentage of GFP+ cells within the entire cell population.

D. Microarray Data analysis

Data normalization was performed using Bioconductor software (v.2.12). The data generated from total RNA samples was normalized using variance stablisation normalization (vsn), based on the work of Huber *et al*. (2002), using a vsn Bioconductor package. For the data originating from hemocyte RNA samples, Loess and Quantile (lquant) normalization was performed, based on the work of Dudoit *et al.* (2002), using the Bioconductor limma software package.

To assess whether the microarray protocol could identify differential expression of genes involved in the immune response between naïve and treated samples, the fold differences in expression for members of immune gene families was calculated. These included effectors, such as AMPs and TEPs, and immune receptors such as PGRPs and GNBPs. Volcano plots were used to analyse the numbers of genes displaying significantly differential expression between naïve and bacterial treated samples, as well as between wounded and non-wounded embryos samples.

Further analysis of the microarray data was performed by Professor S. Dorus, Syracuse University, New York, USA; to discriminate between the effects of PBS and bacterial treatment in embryos, and to assess the further biological relevance of the damage response in laser ablated embryos. Comparisons between microarray data from PBS and Gram-negative injected or Gram-positive injected embryos, or non-wounded and laser ablated embryos were conducted to identify damage and infection specific genes, employing

hierarchical clustering and heat-mapping to group candidates by function. The eBayes function of the Limma software package (Smyth, 2004) was used to adjust the p-values of the microarray data pertaining to laser ablation treatment; to correct for probe-wise sample variance and false discovery rates. Comparisons were also drawn directly between microarray data for Gram-positive and Gram-negative infected embryos, to investigate the degree of overlap of gene expression upon these differential infections, and to identify any potential specific Gram-negative or –positive bacterial response genes. Since n=1 for microarray data pertaining to hemocyte transcriptional profiles upon PBS or Ecc15 treatment, the 100 genes showing greatest up- or down-regulation upon either treatment were selected for further analysis.

Networking analysis was performed on the top 100 genes significantly upregulated upon wounding, as well as the genes demonstrating the greatest modulation in hemocytes upon PBS or *Ecc15* treatment, using the freeware Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (v9.05; von Mering *et al.,* 2004). Maps of predicted interactions were produced, with nodes representing genes and edges the nature of the predicted interaction. Confidence values were also calculated for each predicted edge to indicate the probability of a true biological interaction.

Gene ontology (GO) analyses were also conducted in the comparison of microarray data originating from PBS and bacteria treated embryos, using the Gene Ontology Enrichment Analysis Visualisation Tool (Eden *et al.,* 2007; Eden *et al.,* 2009) to assign biological process terms. GO analyses pertaining to molecular function and cellular component terms were performed using STRING. P-values of the enrichment analysis and q-values to represent the correction of the p-values for multiple testing were generated.

E. Statistical analysis

For mortality assays, CFU determination assays and the 20-HE rescue assay, an unpaired T-Test was used to ascertain statistical significance between control and treated groups.

For analysis of RT-qPCR data pertaining to immune gene expression, a one-way ANOVA with a Tukey's post-test was used to assess statistical significance between control and treated groups, as well as between WT and mutant embryo groups. To analyse RT-qPCR data related to the expression of 20-HE signaling components, the Relative Expression Software Tool (REST) 2009 (Pfaffl 2001; Pfaffl *et al.,* 2002) was used to assess the statistical significance of changes in immune gene expression due to levels of efficiency observed within primer sets. This method accounted for reaction efficiency and reference gene normalization. A hypothesis test was employed to determine the probability of the alternate hypothesis, and hence if the differences observed between control and sample data was due to chance. This method performed a large number of random relocations of samples and controls between the groups and then counted the number of times the relative expression of the randomly assigned group is greater than the sample data. 2000 randomisations were applied to the data in this case.

To assess the statistical significance between the numbers of phagocytosed bacteria in wild-type and $Scar^{\Delta 37}$ mutant embryos, a Mann-Whitney U test was employed.

Chapter 3 – Characterisation of the *Drosophila* embryo as a Model Immune System to Study Live Bacterial Infection *In Vivo*

3.1 Introduction

3.1.1 *Drosophila* embryos as an immunity model

Whilst *Drosophila* adult fly and larval models, as well as mbn-2 and S2 cells, have found increasing application as immunity models to study infection both *in vivo* and *in vitro* with a wide range of pathogenic bacterial species (Tzou *et al.,* 2002; Cheng and Portney, 2003; Needham *et al.,* 2004; Agaisse *et al.,* 2005; Blow *et al.,* 2005), the *Drosophila* embryo has only recently emerged as a potentially viable immunity model. This has encompassed limited preliminary assessment of the embryonic capability to mount systemic and local *Cec* responses upon stimulation with LPS extract (Onfelt Tingvall *et al.,* 2001; Tingvall *et al.,* 2001). Furthermore, Vlisidou *et al.* (2009) assessed the ability of embryonic plasmatocytes to effectively phagocytose bacterial species such as *E. coli* and *P. asymbiotica*; taking advantage of the power of the embryo system for live imaging the early host-pathogen interactions. Additionally, Stramer *et al.* (2008) sought to define the genes that are activated in the *Drosophila* embryo, and specifically in hemocytes, upon damage inflicted by sterile laser ablation. This study elucidated that *Drs* was expressed in the embryo upon wounding, as well as genes such as Growth Arrest and DNA Damage-inducible 45 (GADD45), a stress response gene induced by damage which is able to regulate mitogen-activated protein (MAP) kinase signaling (Takekawa and Saito, 1998), whose activation at the wound site requires hemocytes (Stramer *et al.,* 2008) and is postulated by the authors to have a role in epigenetic regulation of wound targets. Despite these studies, a holistic and in-depth view of the embryonic immune capacity and the mechanisms by which immune responses within the embryo are mediated remain elusive.

Adult and larval immunity models have also allowed for the development of techniques and tools to analyse the immune response in *Drosophila*. These typically focus on AMP gene expression after bacterial challenge, as this provides an effective read-out of the *Drosophila* immune response (Lemaitre and Hoffmann, 2007). For instance, RT-qPCR has been utilized to assess the relative expression of AMPs in gut tissue (Buchon *et al.,* 2009), whole flies and larvae (Bischoff *et al.,* 2006; Zaidman-Remy *et al.,* 2006) and S2 cells (Rus *et al.,* 2013) post infection. Moreover, *Drosophila* AMP reporter lines can be used to monitor the time-course and localisation of the immune response; flies carry a transgenic construct whereby the promoter for a specific AMP is fused to a reporter gene, such as *GFP* or *lacZ* (Nehme *et al.,* 2007; Romeo and Lemaitre, 2008; Gendrin *et al.,* 2009; Junell *et al.,* 2010), hence permitting for the observation of AMP gene expression post infection as shown in Figure 3.1.

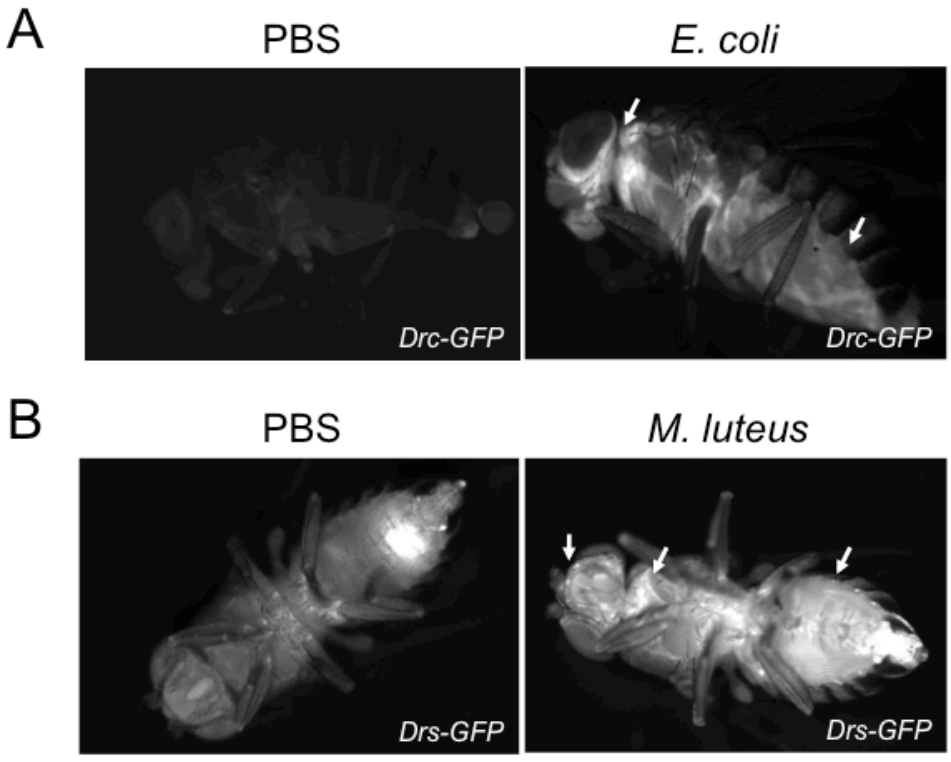


Figure 3.1: Transgenic AMP reporter *Drosophila* lines facilitate the study of immune gene expression upon bacterial stimuli. Traditional infection studies have employed adult flies carrying transgenic promoter-reporter gene fusions to assess the time-course and localisation of immune gene expression post infection. These include the promoters for AMP genes such as *Drc* (**A**) and *Drs* (**B**). The reporter gene in the images displayed is GFP, and upon stimulation with an appropriate microbe, reporter expression can be induced. White arrows indicate areas of reporter expression post infection.

As such, these techniques and resources have the potential for adaptation for use alongside the embryo system. However, to gain novel insight into the *Drosophila* immune response, there is also the potential to employ time-lapse confocal microscopy so as to study the dynamics of host-pathogens interactions *in vivo* and in real-time. This technique has been previously employed within the embryo to study a diverse range of biological processes, such as cell migration, wound healing and inflammation, dorsal ventral patterning, the diffusion of morphogen gradients and the prioritization of damage and developmental signals by hemocytes (Wood *et al.,* 2002; Stramer *et al.,* 2005; Moreira *et al.,* 2010; Sample and Shvartsman, 2010; Siekhaus *et al.,* 2010; Reeves *et al.,* 2012; Evans *et al.,* 2013).

However, traditional routes of infection employed in conjunction with *Drosophila* adult and larval models, such as oral (Kylsten *et al.,* 1990; Vodovar *et al.,* 2005; Liehl *et al.,* 2006; Buchon *et al.,* 2009), topical (De Gregorio *et al.,* 2001; Ha *et al.,* 2005b) or septic injury (Kylsten et al., 1990; De Gregorio et al., 2001; Agaisse *et al.,* 2003; Nehme *et al.,* 2011) routes are not necessarily applicable to the potential *Drosophila* embryo model. For embryo injection, Tingvall *et al.* (2001) adopted an injection procedure using fine glass capillaries, and Vlisidou *et al.* (2009) also favoured an injection procedure, but utilizing a microinjector for a higher level of precision. However, other protocols for the infection of *Drosophila* embryos by permeabilisation with solvents such as hexane and then incubation with bacterial lysates also exist (Esfahani *et al.,* 2011). Esfahani *et al.* (2011) demonstrate that this method of infection was able to stimulate an AMP response in large numbers of *Drosophila* embryos, thus representing a high-throughput method for infecting large numbers of embryos.

3.1.2 Development of the *Drosophila* immune system during embryogenesis

Other than the aforementioned studies, very little is known about the development and capabilities of the immune system within the *Drosophila* embryo, when the system as a whole is activated in concert or how the robust

immune competence exhibited in larvae arises. In terms of fat body development, precursors separate from the inner mesoderm layer at approximately Stage 11 or 12 of development (Hartenstein, 1993; Moore *et al.,* 1998). The column of fat body precursors subsequently form elongated sheets of cells, trapped between the developing visceral musculature and the body wall (Hartenstein, 1993), during Stage 13. Hartenstein (1993) cites that some specializations of the fat body can be observed at this stage, although other studies have demonstrated that the start of terminal fat cell differentiation may occur as early as Stage 12 (Hoshizaki *et al.,* 1994). At Stage 17, it has been noted that large holes and cleft are visible in the previously solid sheets of cells that comprise the fat body structure (Hartenstein, 1993). Moreover, these apertures co-localise with regions where other organs, such as the trachea and gonads, contact the fat body (Hartenstein, 1993). However, the precise timing of fat body maturation to the point where this tissue is fully immune competent to raise a systemic response remains relatively uncharacterized; previous work by Tingvall *et al.* (2001) highlighted that AMPs, such as CecA1, could be induced in fat body tissue in all three stages of larval life, but not at embryonic stages, and lead them to postulate that the fat body requires full maturation during the first instar larval stage of development in order to facilitate successful induction of AMP genes (Tingvall *et al.,* 2001).

Similarly, relatively little is known about the potential for the embryo to mount a local immune response to bacterial infection. Using an embryos that contained a transgenic *CecA1-lacZ* reporter, Onfelt Tingvall *et al*. (2001) reported that embryos were able to activate this construct upon injection with live *Enterobacter cloacae* or LPS extract, and that the response was localized to yolk and epidermal tissue. This expression was noted in Stage 14 embryos, 5 hours after the initial infection (Onfelt Tingvall *et al.,* 2001). Interestingly, injection of bacteria into the perivitelline space resulting in a higher frequency of embryos exhibiting *CecA1-lacZ* expression in the yolk (Onfelt Tingvall *et al.,* 2001), and the authors suggest that this indicates that bacteria must enter the perivitelline space in order to mount a response (Onfelt Tingvall *et al.,* 2001). Moreover, this response in the yolk requires the GATA site, a consensus sequence that has been shown to regulate tissue specificity of AMP gene

expression in larvae (Petersen *et al.,* 1999), whereas the epidermal response was shown to be unaffected by mutation of the GATA site (Onfelt Tingvall *et al.,* 2001). The *serpent* (*srp*) gene, which encodes a GATA-binding transcription factor, is also required for *CecA1-lacZ* expression in yolk, but not epidermal tissue (Onfelt Tingvall *et al.,* 2001). Taken together, these studies suggest that differential transcription regulators are used by the embryo to activate the local immune responses of specific tissues (Onfelt Tingvall *et al.,* 2001).

The development of hemocytes during embryogenesis is vastly better documented (reviewed in Wood and Jacinto, 2007). Both embryonic plasmatocytes and crystal cells develop in the procephalic mesoderm at Stage 10 of embryogenesis. From this position, the first plasmatocytes infiltrate the germ band and are passively carried by germ band retraction to the posterior of the embryo. Further plasmatocytes subsequently leave the head and begin to migrate along the dorsal side of the embryo. Subsequently, populations of plasmatocytes from the anterior and posterior regions of the embryo migrate towards one another along the embryonic midline via the ventral nerve cord, and the developing gut until they meet at Stage 14. At Stage 15, the plasmatocytes migrate laterally, leaving the embryonic midline, and subsequently their migration then becomes random. This plasmatocyte migration is invariant (Wood and Jacinto, 2007), and thus can be observed in all wild-type *Drosophila* embryos. During this stereotypical migration, embryonic plasmatocytes engage in many crucial developmental functions, including the clearance of apoptotic cells throughout the embryos via phagocytosis and the secretion of extracellular matrix proteins (Wood and Jacinto, 2007). There is also a requirement of plasmatocytes for the correct development of organs such as the gut and central nervous system (Olofsson and Page, 2005). Few studies have addressed the immune capacity of embryonic plasmatocytes. Work by Vlisidou *et al.* (2009) demonstrated that embryonic plasmatocytes at Stage 15 of development do not actively migrate towards to invading bacteria but opportunistically recognize, bind and phagocytose the pathogens as they are washed over their surfaces in extracellular space. However, although these hemocytes exhibit effective

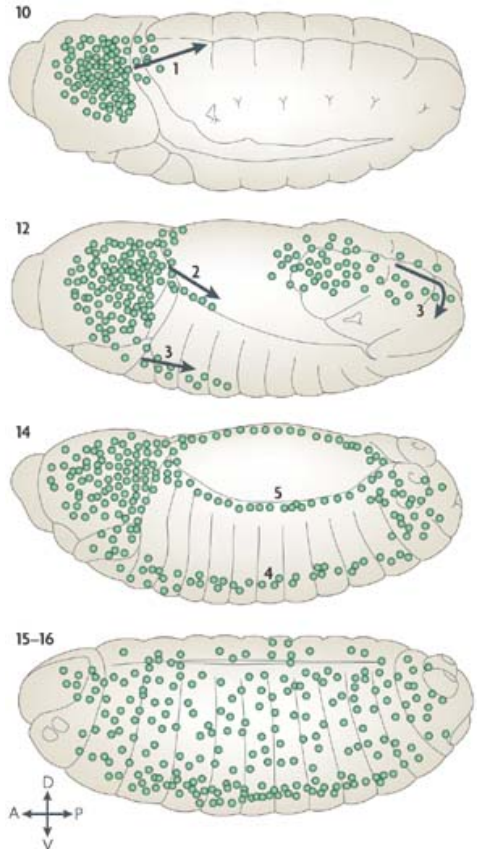


Figure 3.2: Developmental migrations of Drosophila embryonic hemocytes, taken from Wood and Jacinto (2007). *Drosophila* embryonic hemocytes first develop in the head mesoderm, subsequently migrating along stereotypical routes to populate the embryo. Hemocytes leave the head mesoderm at Stage 10 of embryogenesis and infiltrate the germ band (1). At Stage 12, germ band retraction transports these hemocytes towards the posterior of the embryo, whilst further hemocytes leave the anterior region and migrate along the dorsal side of the embryo (2). Hemocytes from the anterior and posterior of the embryo subsequently migrate along the ventral nerve cord (3) until these two populations meet at Stage 14 (4). Hemocytes continue to migrate along the dorsal side of the embryo, along the developing dorsal vessel (5). At Stage 15-16, hemocytes disperse from this stereotypical pattern, and begin to migrate in a random fashion, becoming evenly distributed over the embryo. Compass represents embryo parameters: A, anterior; P, posterior; D, dorsal; v, ventral.

mechanisms to eliminate invading pathogens, they are not immune to virulence factors secreted by the infection itself. Infection of Stage 15 embryos with a Gram-negative insect pathogen, *Photorhabdus asymbiotica*, was shown to rapidly halt the migration of plasmatocytes via freezing cellular protrusions and phagosomes, permitting bacteria to escape phagocytosis (Vlisidou *et al.,* 2009). This effect was attributed to the cellular internalization of the *P. asymbiotica* toxin Makes Caterpillars Floppy 1 (Mcf1), potentially involving the activity of Rac GTPase (Vlisidou *et al.,* 2009). Conversely, differentiated crystal cells form a small cell group that remains clustered around the

embryonic proventriculus (Lebestky *et al.,* 2000). Whilst limited studies focusing on the response of embryonic plasmatocytes upon infection have been conducted, no role has yet been suggested for embryonic crystal cells under these conditions, thus the function that they play in this process, if any, has yet to be determined.

3.1.3 Regulation of innate immunity via steroid hormone signaling

Aside from the canonical immune pathways that centre on the Toll-like and TNF signaling cascades, steroid hormone and their respective receptors have relatively recently been implicated in the regulation of the adaptive and innate immune responses in mammalian systems (Chow *et al.,* 2006). For example, the glucocorticoid receptor (GR) has been extensively demonstrated to repress pro-inflammatory genes, potentially via GR binding to and activation of IκBα, thus sequestering the NFκB subunit proteins p50/p65 and preventing them from initiating the transcription of pro-inflammatory gene targets (Chow *et al.,* 2006). However, GR has also been shown to directly associate with p65, thus potentially acting as a steric inhibitor and preventing the transcription factor's interaction with DNA (McKay and Cidlowski, 2000). However, GR is not exceptional; many other mammalian steroid hormone receptors have been linked to pro-inflammatory gene repression and AMP induction, such as the oestrogen receptor (ER), vitamin D receptor (VDR), liver X receptor (LXR), retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) (Tontonoz *et al.,* 1998; Gombart *et al.,* 2005; Hong and Tontonoz, 2008; Jetten, 2009; Baeke *et al.,* 2010; Nunez *et al.,* 2010). Moreover, this mechanism of immune regulation is not restricted to mammalian systems, but is a common theme throughout the animal kingdom; for instance, Aguila *et al.* (2013) highlighted the importance of testosterone and 11-ketotestosterone in the regulation of professional phagocyte responses, such as phagocytosis, to the fish pathogen *Vibrio anguillarum* in seabream, whilst Watanuki *et al.* (2002) demonstrated the effects of steroids including β-oestradiol and progesterone on the negative regulation of phagocytosis, superoxide and nitric oxide production in the common carp *Cyprinus carpio*.

Drosophila is not an exception to this trend. One such hormone in *Drosophila* that has been implicated in the modulation of innate immunity is 20-hydroxyecdysone (20-HE). 20-HE is a polyhydroxylated steroid signaling molecule (Schwedes and Carney, 2012) that has been shown to control insect molting, metamorphosis and other developmental processes via its coordinated release into the circulating hemolymph from the prothoracic gland, a component of the ring gland, (Pankotai *et al.,* 2010). Whilst 20-HE has traditionally been implicated in regulating a range of other developmental processes such as reproduction (Carney and Bender, 2000; Schwedes and Carney, 2012), lifespan and aging (Simon *et al.,* 2003; Simon *et al.,* 2006; Tricoire *et al*., 2009), as well as adult fly behaviour (Ishimoto *et al.,* 2009; Ishimoto and Kitamoto, 2010; Ganter *et al.,* 2011), more recent studies have provided evidence that this steroid hormone may also be crucial for immune competence, and hence play a role in regulating the *Drosophila* immune response. For instance, a plethora of *in vitro* studies have suggested that 20-HE may play a role in modulating both the systemic and cellular responses. It was noted that when using S2 cells for immunity studies that 20-HE application further enhanced the AMP response *in vitro* upon a bacterial stimulus, as well as altering cell morphology and adherence (Silverman *et al.,* 2003; Flatt *et al.,* 2008). This was mirrored by results from Dimarcq *et al.,* (1997), where the authors used cultured mbn2 cells to show that treatment with 20-HE induced a differentiation to a more adherent, macrophage-like morphology in this cell line, resulting in an increased phagocytic capacity and enhanced ability to express AMP genes, as well as other immune genes such as those encoding Scavenger receptors, in response to infection or a bacterial elicitor such as PGN. More specific evidence of steroid hormone modulation of *Drosophila* systemic immunity was presented by Zhang and Palli (2009); deletion analysis in mbn2 cells identified a *cis*-regulatory element within the *Drosophila Dpt* promoter that enabled enhanced expression upon 20-HE binding after exposure to *E. coli* PGN. 20-HE was also shown to further enhance the PGN-induced expression of other AMP genes, including *Drs, AttA, Mtk* and *CecA1* (Zhang and Palli, 2009). In particular, an 8-nucleotide region of the element discovered to be present in the promoter regions for

each of these AMP genes using *in silico* promoter analysis (Zhang and Palli, 2009). However, the precise mechanism by which these 20-HE driven AMP responses were mediated remained elusive until the recent discovery by Rus *et al.* (2013) that 20-HE is able to regulate signal transduction via the IMD pathway. Microarray analysis demonstrated that expression of several IMD signaling components such as *dIAP2*, *Tab2*, *Relish* and *Caspar* could be moderately and significantly induced upon 20-HE stimulation of S2 cells (Rus et al., 2013). More interestingly, the expression of *PGRP-LC* was robustly induced upon 20-HE treatment of S2 cells, and 20-HE was subsequently demonstrated to control AMP gene induction via two distinct mechanisms (Rus *et al.,* 2013). The initial mechanism presented in this work involved the 20-HE regulated expression of PGRP-LCx, which was able to drive induction of a subset of AMP genes, such as *CecA1*, *AttA* and *Def* (Rus *et al.,* 2013). However, the actions of 20-HE in this mechanism could be bypassed by the ectopic expression of PGRP-LC itself (Rus *et al.,* 2013). The second mechanism described involves the expression of a further subset of AMPs, for which 20-HE activity is critically required and cannot be bypassed via ectopic *PGRP-LC* expression, including *Dpt*, *Mtk* and *Drs*. This level of regulation is proposed by Rus *et al.* (2013) to act downstream of Relish, involving classical 20-HE transcriptional targets. A third mechanism of AMP regulation by 20-HE was postulated by Flatt *et al.* (2008), whereby findings elucidated that the 20-HE promotion of AMP gene expression may be antagonized by the action of Juvenile Hormone (JH), another *Drosophila* steroid hormone, which seemingly acts as an immunosupressor (Flatt *et al.,* 2008). What is not immediately clear is how these complex mechanisms interact to modulate the overall systemic response.

Nevertheless, the findings of these *in vitro* studies have subsequently been partially confirmed in an *in vivo* context. For example, in the context of the cellular response, Sorrentino *et al.* (2002) observed a dramatically compromised encapsultion response to wasp parasitization in 3rd instar larvae with low 20-HE titres, both controlled genetically by the *ecdysoneless* mutation and when 20-HE signaling was blocked. This was accompanied by decreases in crystal cell maturation and lamellocyte differentiation post infection

(Sorrentino *et al.,* 2002), suggesting that 20-HE signaling is required for a complete anti-parasitic response. In a similar vein, Lanot *et al.* (2001) established that plasmatocyte phagocytic activity could be enhanced by injecting 3rd instar larvae with 20-HE, highlighting the hormone's ability to regulate the cellular response. In terms of systemic immunity, further studies in *Drosophila* larvae have highlighted that the immune competency of fat body cells increases in larval stages under the control of 20-HE signaling (Meister and Richards, 1996); a further hint that 20-HE is able to modulate systemic immunity *in vivo*. Finally, Rus *et al.* (2013) examined the immune role of 20-HE signaling components *in vivo* using adult flies. Depletion of 20-HE signaling components and effectors resulted in the decreased expression of *Dpt* and *CecA1*, as well as *PGRP-LC*, upon *E. coli* infection, demonstrating that 20-HE signaling can regulate IMD pathway activity *in vivo* either via the direct effects of 20-HE or via 20-HE signaling effectors (Rus *et al.,* 2013). Taken together, these results suggest that both the systemic and cellular immune responses in *Drosophila* are regulated via 20-HE signaling. In some cases, 20-HE may exert its effects through interaction with the IMD pathway, particularly in relation to AMP induction, although how 20-HE may mediate its effects in other immune processes, such as the cellular response, remains unclear.

3.1.4 20-HE signaling components and effectors

The production of 20-HE is known to be a complex process, which involves a multitude of biosynthetic enzymes and the cellular transport machinery (Gilbert *et al.,* 2002; Lafont *et al.,* 2004). In the 20-HE biosynthesis in many insect models, dietary cholesterol is dehydrogenated in the endoplasmic reticulum and then transported to the mitochondria for oxidation (Lafont *et al.,* 2004). Subsequently, sequential production of diketol, ketodiol and ketotriol intermediates occurs, under the control of Black Box enzymes and hydroxylases, before the release of inactive ecdysone into the hemolymph and the final ensuing modification to its active form, 20-HE at target tissues (Gilbert *et al.,* 2002; Neubueser *et al.,* 2005). However, despite the characterization of many steroidogenic enzymes in a range of insect species, the biosynthesis of

20-HE at the molecular level in *Drosophila* is relatively poorly understood. However, recent work has attempted to address this deficit. It is now established that the steroidogenic hydroxylases involved in *Drosophila* 20-HE biosynthesis are Cytochrome P450 enzymes, which are encoded by members of the so-called Halloween family of genes (Neubueser et *al.,* 2005), as depicted in Figure 3.3. The Halloween family members involved in ecdysone biosynthesis include *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*), which are required for the synthesis of pathway intermediates in the prothoracic gland cells of the larval ring gland (Neubueser *et al.,* 2005). *Phm* encodes a hydroxylase which converts ketodiol to ketotriol in the intermediate steps of ecdysone synthesis, whereas *dib* and *sad* encode 22C-hydroxylating and 2C-hydroxylating enzymes respectively which further modify ketotriol to an inactive form of ecdysone (Neubueser *et al.,* 2005). Inactive ecdsyone is subsequently released into the hemolymph and it is converted to its active form, 20-HE, at its target peripheral tissues by the 20-monooxygenase Shd (Petryk *et al.,* 2003). The transcription of the Halloween genes itself is controlled by Prothoracicotropic hormone (Ptth), a neuropeptide whose release from the brain signals through the receptor Torso to activate MAPK signaling and stimulate activity of the prothoracic gland (McBrayer *et al.,* 2007; Rewitz *et al.,* 2009; Yamanaka *et al.,* 2013). Moreover, regulation of Halloween protein activity may also stem from their requirement of cofactors. Each of the Halloween hydroxylases requires an electron pair from NADPH to modify their substrates (Neubueser *et al.,* 2005). A recently identified 20-HE biosynthesis component, termed Defective in Avoidance of Repellents (Dare), may indirectly play a role in this process. Dare is known to be a close homologue of vertebrate adrenodoxin reductase (Freeman *et al.,* 1999), which transfers electrons from NADPH to adrenodoxin proteins. In turn, adrenodoxin donates electrons to steroidogenic cytochrome P450 hydroxylases required for ecdysone synthesis (Kozlova and Thummel, 2000). As such, it has been suggested that Dare may play a similar role in *Drosophila*. Concordant with this hypothesis is the fact that *dare* expression is enriched in the larval ring gland (Kozlova and Thummel, 2000) the *Drosophila* endocrine organ responsible for ecdysone production, and *dare* mutant larvae exhibit defects in molting and pupariation (Freeman *et al.,* 1999); developmental processes

which require ecdysone signaling. Moreover, there is evidence that other genes, such as *molting defective* (mld), *ecdysoneless* (*ecd*) and *neverland* (nvd), may encode other factors that are required for 20-HE biosynthesis, but their precise molecular roles remain speculative (Gaziova *et al.,* 2004; Neubueser *et al.,* 2005; Yoshiyama *et al.,* 2006).

The resulting active 20-HE is then free to bind to its corresponding heterodimeric receptor complex, consisting of the proteins Ecdysone Receptor (EcR) and Ultraspiricle (Usp) (Yao *et al.,* 1992; Thomas *et al.,* 1993; Yao *et al.,* 1993); these are orthologs of the mammalian liver X and retinoid X receptors respectively (King-Jones and Thummel, 2005). In the absence of 20-HE, EcR is heterogeneously distributed between the nucleus and cytoplasm (Nieva *et al.,* 2005). Heterodimerisation with Usp stimulates the receptor complex's nuclear transport, where Usp transfers EcR quantitatively into the nucleus (Nieva *et al.,* 2005), reminiscent of the recruitment of nuclear recruitment of the Vitamin D receptor in mammalian systems (Prüfer *et al.,* 2000). Once inside the nucleus, EcR-Usp binds to DNA sequences, known as Ecdysone Responsive Elements (EcREs) to facilitate the transcription of downstream genes (Schwedes *et al.,* 2011). However, other evidence suggests that when 20-HE titres are low, EcR-Usp can also exist as an unliganded heterodimer bound to EcREs to promote the repression of transcription (Schwedes *et al.,* 2011).

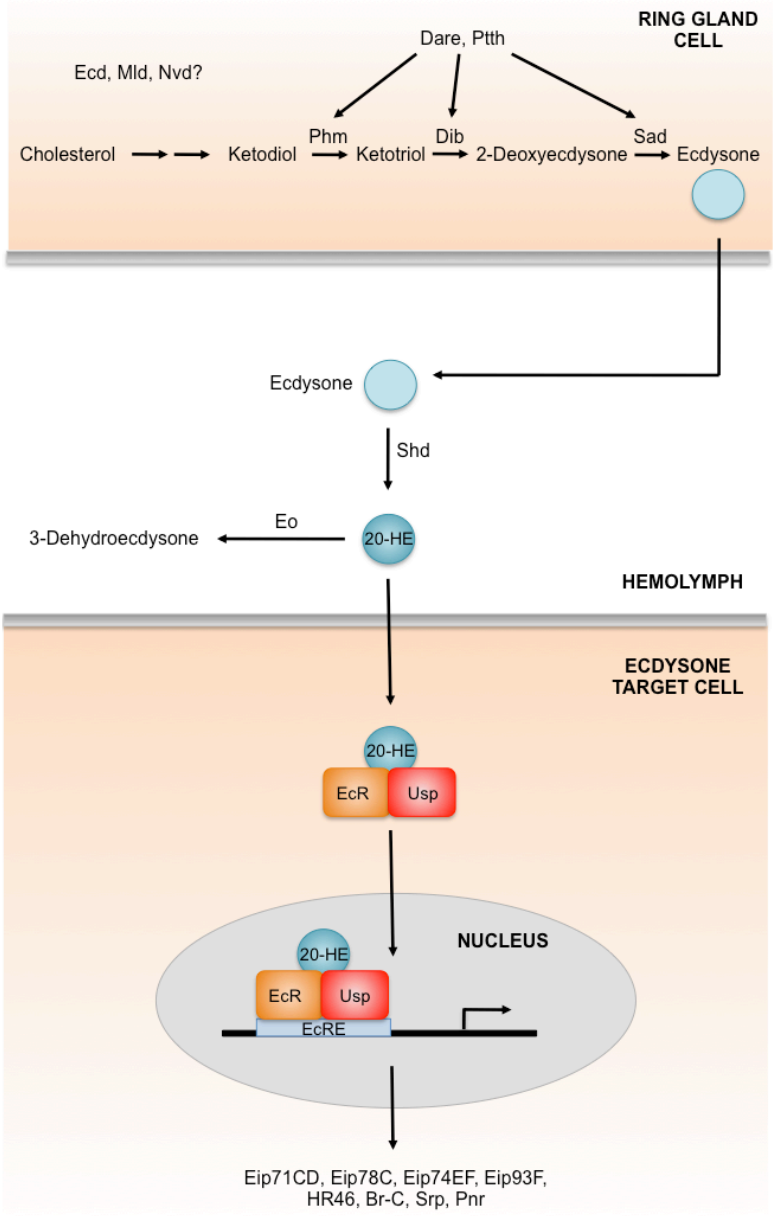


Figure 3.3 Schematic of 20-Hydroxyecdysone Signaling in *Drosophila*. Cholesterol is converted to the inactive form of ecdysone within the prothoracic cells of the ring gland, via the production of ketodiol, ketotriol and 2-deoxyecdysone intermediates by Halloween gene products Phantom (Phm), Disembodied (Dib), and Shadow (Sad). The activity and transcription of the Halloween genes themselves are controlled by Defective in Avoidance of Repellents (Dare) and Prothoracicotropic hormone (Ptth) respectively. Inactive ecdysone is secreted from the ring gland and converted to its active form, 20-hydroxyecdysone (20-HE) by Shade (Shd) at the target tissue. 20-HE is then free to bind the Ecdysone Receptor (EcR)-Ultraspiricle (Usp) heterordimer receptor complex. The liganded complex translocates to the nucleus where it binds with high affinity to elements in DNA, termed Ecdysone Responsive Elements (EcREs), which facilitates the transcription of downstream genes, such as Ecdysone Inducible Proteins (Eips) 71CD, 78C, 74EF and 93F, as well as other ecdysone responsive genes such as HR46, the Broad complex (Br-C), Serpent (Srp) and Pannier (Pnr). To ensure that ecdysone signaling is effectively switched off to properly regulate developmental processes, 20-HE is metabolized by Ecdysone oxidase (Eo) to 3-Dehydroecdysone.

Upon subsequent ligand binding, EcR-Usp undergoes a conformational change that allows the complex to bind EcREs with greater affinity and recruit coactivators to initiate gene expression (Yao *et al.,* 1992; Yao *et al.,* 1993). Regardless of the mechanism of EcRE binding, the actions of EcR-Usp initiate a highly regulated and temporal pattern of gene activation; some genes are activated within minutes of 20-HE exposure, hence are termed 'early' response genes, whereas others are activated within hours of this event, and thus are given the status of 'delayed early' response genes (Huet *et al.,* 1995; Lan *et al.,* 1999). It has thus been hypothesized that this sequential gene activation represents a complex transcriptional hierarchy, to ensure appropriate responses and developmental processes are correctly mediated in specific target tissues (Ashburner *et al.,* 1974; Thummel, 1995; Chavez *et al.,* 2000).

The *EcR* gene itself was demonstrated to give rise to three distinct EcR isoforms by Talbot *et al.* (1993); the authors consequently clarified that all three isoforms have common DNA- and hormone-binding domains, but differ in their N-terminal regions (Talbot *et al.,* 1993). These isoforms were accordingly termed EcR-A, EcR-B1 and EcR-B2 (Talbot *et al.,* 1993) and are all able to form heterodimers with Usp (Yao *et al.,* 1992; Talbot *et al.,* 1993; Thomas *et al.,* 1993; Yao *et al.,* 1993), hence facilitating downstream signaling events. It is postulated that each isoform subsequently directs specific responses to 20-HE, potentially explaining each receptor's ability to activate or repress gene transcription and how 20-HE is able to drive such a diverse range of tissue and stage specific responses (Mouillet *et al.,* 2001; Schwedes *et al.,* 2011). Analysis of the function of specific EcR isoforms indicates that EcR-A is a weak activator and strong repressor of transcription, whereas the converse is true for EcR-B1 and –B2 (Hu *et al.,* 2003). In larval and pupal tissues, the EcR isoforms are present in a diverse range of tissues, with isoform-specific tissue distributions spatially correlating with developmental fate (Robinow *et al.,* 1993; Talbot *et al.,* 1993; Truman *et al.,* 1994; Bender *et al.,* 1997; Schubiger *et al.,* 1998). Whilst the EcR-B1 and –B2 isoforms have been demonstrated to play crucial roles in larval molting and neuronal remodeling during metamorphosis (Schubiger *et al.,* 1998) and are typically

expressed in larva-specific tissues (Watanabe *et al.,* 2010), the EcR-A isoform is expressed in proliferative tissues during metamorphosis and is required for adult-specific developmental processes (Watanabe *et al.,* 2010), such as oogenesis (Carney and Bender, 2000).

Binding of 20-HE ligand to the EcR-Usp complex regulates the expression of early response genes, many of which are also transcription factors (Rus *et al.,* 2013). These include the Ecdysone-induced protein (Eip) 74EF, an E-Twenty six domain factor, and Eip71CD, a methionine S-oxide reductase that has been implicated in the response to oxidative stress and autophagic cell death (Gorski *et al.,* 2003). Several other Eip genes are subsequently activated later, as a subset of the delayed-early response genes, such as Eip78C; a nuclear hormone receptor that further regulates the 20-HE-mediated transcriptional hierarchy (King-Jones and Thummel, 2005). Moreover, recent work by Rus *et al.* (2013) demonstrated that some of these effectors are critical for signal transduction and induction of AMP genes via the IMD pathway. These include the early inducible transcription factors Eip93F, Eip74EF, Eip78C, Hormone Receptor-like in 46 (Hr46) and the Broad complex (Br-C), as well as two GATA factors, Serpent (Srp) and Pannier (Pnr) (Rus *et al.,* 2013). Interestingly, Eip74EF, Eip78C and Eip75B expression was significantly reduced in 3rd instar larvae in response to localized tissue damage (Hackney *et al.,* 2012).

To ensure that ecdysone production is correctly coordinated and in order to prevent prolonged exposure to 20-HE, the temporal inactivation of 20-HE to ensure correct developmental transitions is vital. One route of metabolism entails the oxidation of 20-HE into 3-dehydroecdysteroid by Ecdysone Oxidase (Eo) (Takeuchi *et al.,* 2005). The activity of *Drosophila* Eo was tested *in vitro* using COS7 cells, where conversion of 20-HE into 3-dehydroecdysone was noted (Takeuchi *et al.,* 2005). Furthermore, Takeuchi *et al.* (2005) demonstrated that Eo was expression predominantly in midgut of late larval instars, temporally equivalent to the 20-HE titre peak, suggesting that Eo may negatively regulate the ecdysone pulses observed in larvae and adult flies.

3.1.5 20-HE signaling during embryogenesis

Via the aforementioned signaling mechanism, 20-HE is crucial for correct development in *Drosophila*. High 20-HE titres, or pulses, occur throughout the *Drosophila* life cycle, as depicted in Figure 3.3, regulating a range of diverse processes, such as the apoptosis of obsolete larval tissues, molting, puparium formation and metamorphosis (Kozlova and Thummel, 2003). One such pulse occurs during mid-embryogenesis (Figure 3.4A, red box and Figure 3.4B), which peaks during germ band retraction at approximately 9 hours after egg-laying (Maroy *et al.,* 1988; Kozlova and Thummel, 2003). During this period of time, 20-HE is required for successful germ band retraction and head involution (Maroy *et al.,* 1988). Furthermore, 20-HE has also been demonstrated to play a role in cuticle deposition during late embryogenesis (Hoffmann and Lagueux, 1985; Chavez *et al.,* 2000). The critical requirement for 20-HE to regulate these processes is supported by the fact that embryos carrying zygotic mutations in *EcR* die during embryogenesis (Bender *et al.,* 1997) and germline clones of *EcR* null mutants are subsequently arrested during oogenesis and thus result in female sterility (Buszczak *et al.,* 1999). These results are not surprising, given that components of the 20-HE signaling pathway such as EcR and Usp are widely expressed by mid-embryogenesis, with activation of the EcR-Usp receptor complex at Stage 13 of development in the amnioserosa (Kozlova and Thummel, 2003). However, relatively little is known about the precise function of 20-HE at early stages of embryogenesis as *EcR* transcript and protein are deposited maternally, making study intrinsically difficult (Talbot *et al.,* 1993). Moreover, the location of 20-HE biosynthesis is not well defined in the *Drosophila* embryo. Studies have suggested that the embryonic epidermis may be a source of 20-HE during mid-embryogenesis, as expression of *sad* and *dib* is concentrated within individual epidermal segments at this time-point, coinciding with the 20-HE embryonic pulse (Warren *et al.,* 2002). Warren *et al.* (2002) further demonstrate that this pattern then diminishes and, with *sad* and *dib* expression subsequently concentrated within the ring gland during late embryogenesis, suggesting a switch in the localisation of 20-HE synthesis. Nevertheless, in light of the evidence that 20-HE is able to regulate innate immunity in

Drosophila, its contribution to the embryonic immune capacity remains uncharacterized.

3.1.6 Experimental aims

Before the *Drosophila* embryo model could be employed in any novel dynamic imaging studies as defined in Vlisidou *et al.* (2009), it was important to fully characterise the model system; to fully understand and validate its immune capacity. Within this broad aim, it was investigated whether *Drosophila* embryos were able to mount both systemic and cellular responses to infection and the mechanisms by which these responses were mediated. Moreover, the system was subsequently utilized to gain an understanding of how immune competence originates within the *Drosophila* embryo and at what point during embryogenesis this occurs. Since 20-HE signaling has such crucial roles in embryogenesis and has been implicated in regulating innate immunity in *Drosophila*, it was investigated whether this form of signaling is required for the embryonic immune response.

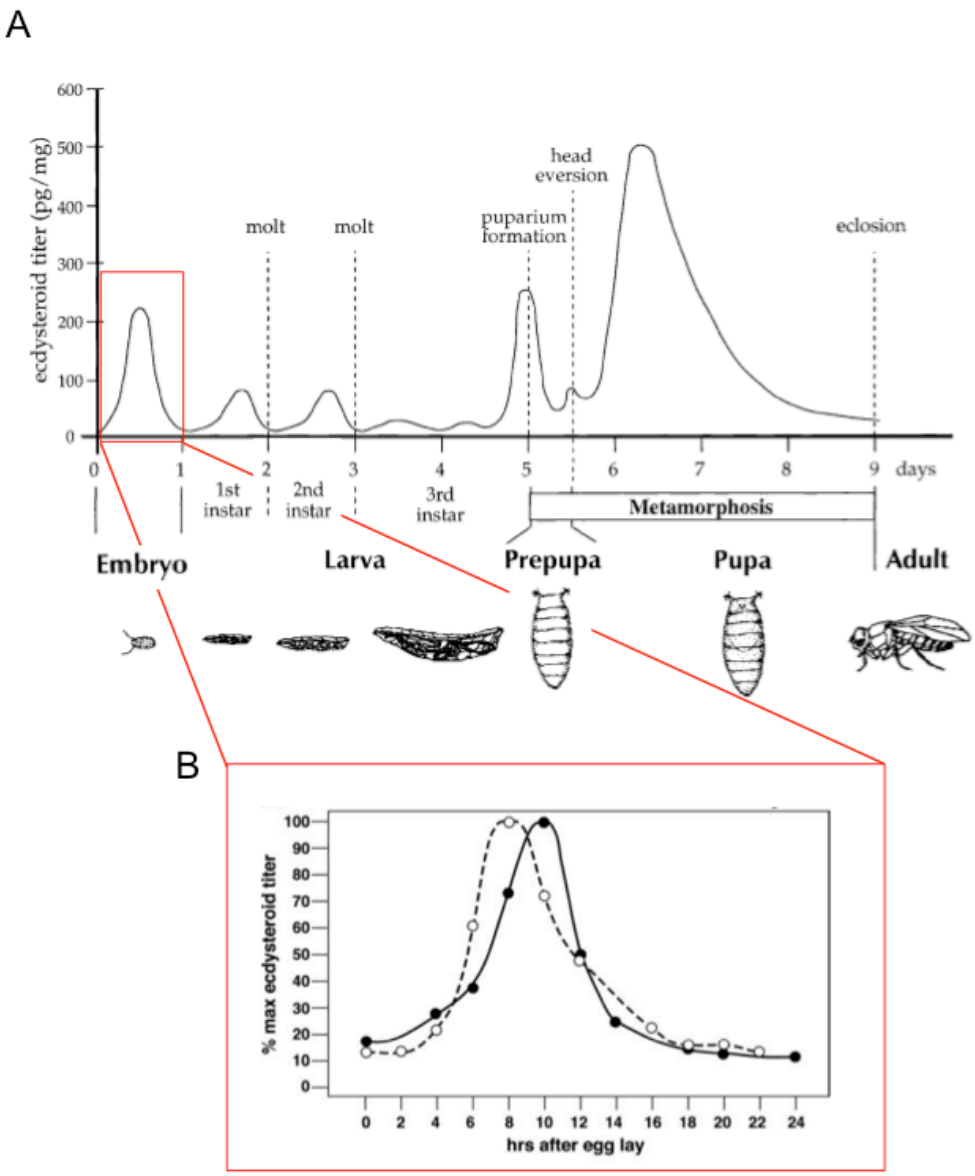


Figure 3.4: 20-HE titres throughout the *Drosophila* lifecycle. 20-HE titres fluctuate dramatically over the *Drosophila* lifetime (**A**; taken from Thummel, 2001), with pulses of 20-HE coinciding with crucial developmental transitions such as molting, puparium formation, and metamorphosis, as indicated by dotted lines. Regarding 20-HE titres during embryogenesis (B; taken from Sullivan and Thummel, 2003), a 20-HE pulse is observed to occur during mid-embryogenesis at approximately Stage 12 of development, at x hours after egg laying. Two different sets of 20-HE titre measurements from staged embryos are plotted from Maroy *et al.* (1988) and Kraminsky *et al.* (1980), in white and black points respectively.

3.2 Results

3.2.1. Stage 15 embryos are able to mount a robust AMP response to bacterial infection

As relatively little was known about the immune capacity of the Drosophila embryo, it was initially necessary to determine whether *Drosophila* embryos were able to mount a robust AMP response to bacterial infection. Focusing on AMP production post infection gave an effective indication of the immune potential of the embryo model system; the activation of these genes has previously been used in both adult fly and larval models to provide an accurate read-out of the systemic immune response, in terms of response time-course and magnitude (Lemaitre *et al.*, 1997). Using a previously established embryo microinjection assay (Vlisidou *et al.,* 2009), Stage 15 embryos containing a *Drosocin-GFP* promoter-GFP fusion construct (*Drc-GFP*) were injected with either live or heat-killed *DH5α E. coli* (OD=1), to determine if AMP gene induction could be induced within the embryo upon a bacterial stimulus. The resulting *Drc-GFP* expression was compared to that of control PBS-injected Stage 15 embryos; to further elucidate if tissue damage inflicted by the microinjection process itself had any effect on *Drc* expression.

Results showed that Stage 15 *Drosophila* embryos were able mediate a robust immune response to *DH5α E. coli* infection (Figure 3.5). Extensive expression of the *Drc-GFP* construct was observed in Stage 15 embryos 6 hours post infection with either live or heat-killed *DH5α E. coli* (Figure 3.5B and C), in contrast to those which received only a control sterile PBS injection (Figure 3.5A). The most intense activation of the *Drc*-GFP construct was noted with live *E. coli* infection (Figure 3.5B). However, the percentage of embryos demonstrating a *Drc-GFP* response post injection with either live or heat-inactivated *DH5α E. coli* was not significantly different, suggesting that the presence of live bacteria is not strictly required for AMP induction in the Stage 15 embryo (Figure 3.5H). This phenomenon was not observed solely with *DH5α E. coli* infection, as injection of Stage 15 embryos

with other strains of *E. coli*, such as the *Mg1655 E. coli* strain (Figure 3.5D), and other Gram-negative species such as *TTO1 Photorhabdus luminescens* (Figure 3.5E) and *Erwinia carotovora carotovora 15* (*Ecc15*; Figure 3.5F) also induced extensive *Drc-GFP* expression 6 hours post infection. Injection with *Salmonella typhimurium* elicited a less robust response, with only minor expression noted at the same time point (Figure 3.5G).

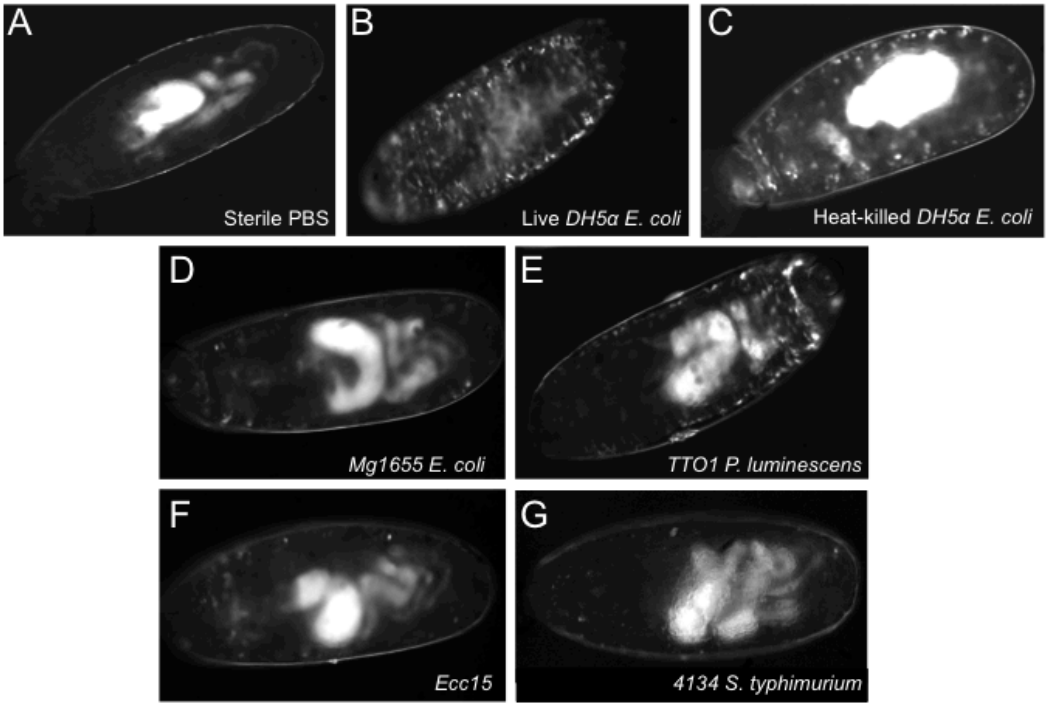
To further confirm these results and to investigate the relative expression levels of a range of AMP genes upon *DH5α E. coli* infection, RT-qPCR studies were performed on infected Stage 15 embryos at 2 hours post injection (Figure 3.6A-B). These experiments focused on the Gram-negative AMP genes *Dpt*, and *CecA1*. Relative levels of gene expression were calculated with reference to the internal control gene, *rp49*, and the fold difference in AMP gene expression between treatments was calculated by normalizing values to those of non-injected (NI) control groups. Relatively low basal levels of individual AMP expression were observed in NI Stage 15 embryos (Figure 3.6A-B). Upon injection with *DH5α E. coli*, relative expression levels of *Dpt* and *CecA1* genes demonstrated significant increases by 35- and 50-fold respectively ($p \leq 0.01$, T-Test).

Furthermore, it was revealed that bacterial infection itself was not the only stimulus of AMP gene expression within the microinjection assay. Expression of AMP genes within the PBS-injected embryos also exhibited a significant relative increase from non-injected basal levels (Figure 3.6A-B). Whilst the induction of the AMP genes under these conditions were not concordant with those levels observed in *DH5α E. coli*-injected embryos, it was clear that Stage 15 embryos could nevertheless induce a relatively lower level of AMP expression in response to damage inflicted via the microinjection process itself. Interestingly, in the case of *CecA1* (Figure 3.6B), the levels of expression upon PBS injection were observed to be comparable to those in *DH5α E. coli*-injected embryos, with no significant difference in expression noted between these treatment groups (Figure 3.6B). This may suggest that some individual AMP genes may be more responsive to upstream damage

signaling and are more highly expressed under these conditions. Taken together, these results confirm that Stage 15 *Drosophila* embryos are able to mount a robust systemic response to bacterial challenge, as well as damage, consisting of the induction of multiple AMP genes.

Figure 3.5: Stage 15 *Drosophila* embryos are able to mount a robust *Drc-GFP* response to Gram-negative bacterial challenge.

(**A-C**) Stage 15 *Drc-GFP* embryos were microinjected with sterile, endotoxin-free PBS (**A**), live (**B**) or heat-killed (**C**) *DH5α E. coli* to assess the immune potential of *Drosophila* at this stage of embryogenesis. Whilst no response was noted at 6 hours post infection in embryos injected with PBS, those that received *E. coli* inoculum demonstrated extensive expression of *Drc-GFP*, with live *E. coli* producing the most intense response. (**D-G**) This response is not limited to *DH5α E. coli* infection, as *Mg1655 E. coli* (D), *TTO1 P. luminescens* (E) and *Ecc15* (F) also promoted a similar *Drc-GFP* response. Infection with *4134 S. typhimurium* induced a concordant, but weaker response (G). (**H**) Quantification of the percentage of *Drc-GFP* expressing embryos upon sterile endotoxin-free PBS, *DH5α E. coli* or heat-inactivated *DH5α E. coli* treatment. Injection of both live and heat-inactivated *DH5α E. coli* significantly increased the percentage of responding embryos compared to PBS control treatment, but heat-inactivated *DH5α E. coli* injection stimulated significantly fewer embryos than live bacterial infection (Unpaired T-test, n=3 with ≥25 embryos injected per experiment; * Indicates $p \leq 0.05$, *** indicates $p \leq 0.001$. Error bars represent standard deviation).


A
Sterile PBS
B
Live DH5α E. coli
C
Heat-killed DH5α E. coli
D
Mg1655 E. coli
E
TTO1 P. luminescens
F
Ecc15
G
4134 S. typhimurium

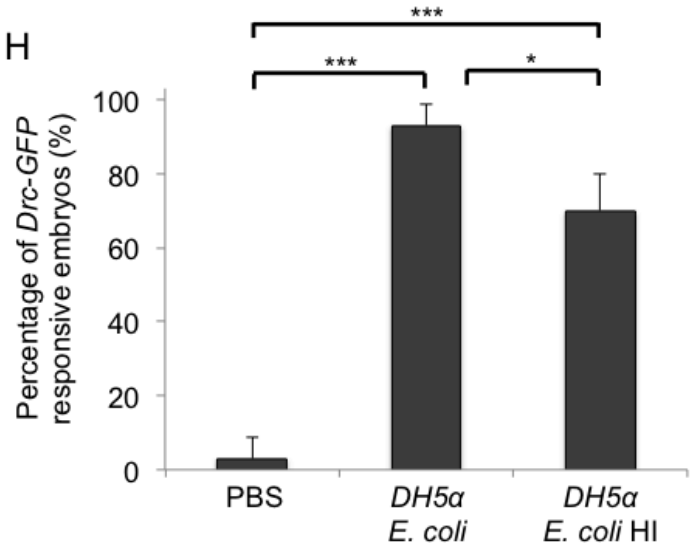
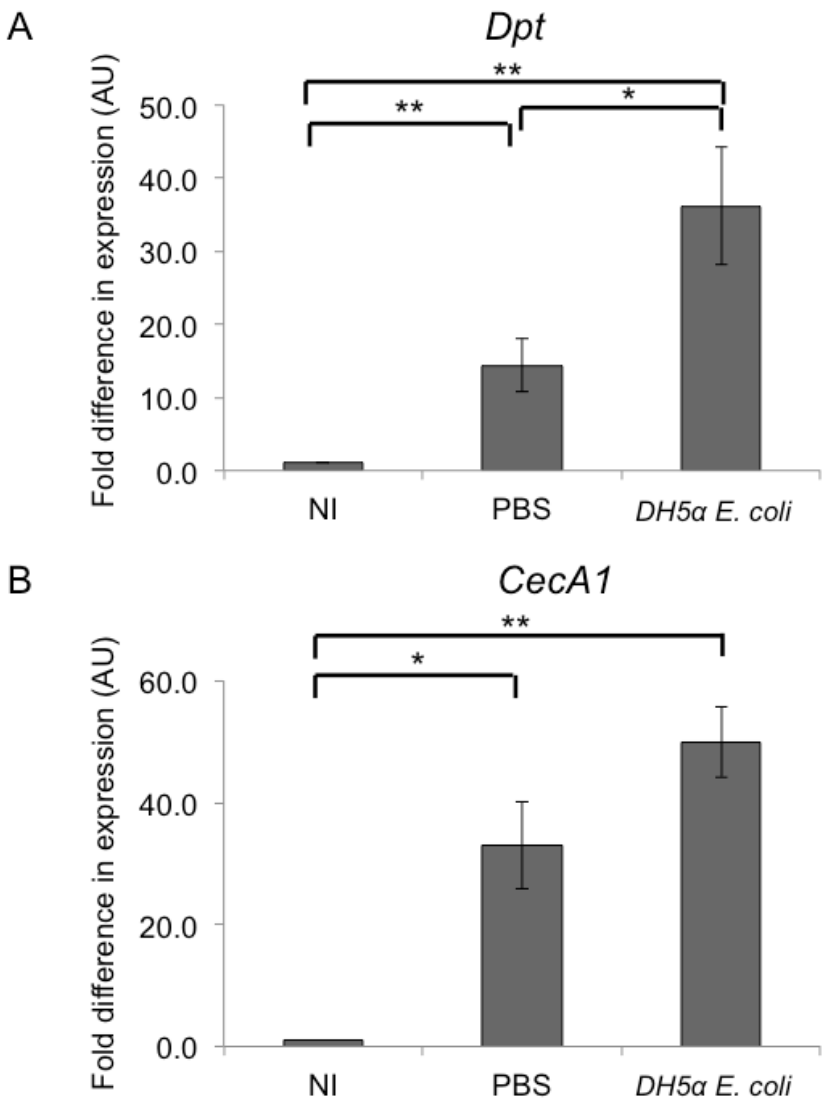

H
***
***
*
Percentage of Drc-GFP responsive embryos (%)
100
80
60
40
20
0
PBS
DH5α E. coli
DH5α E. coli HI

Figure 3.6: Stage 15 *Drosophila* embryos are able to induce AMP gene expression upon bacterial infection.

(**A-B**) Bar graphs showing the fold difference in expression of four antimicrobial peptide genes upon injection with PBS or *E. coli.* Expression levels in injected embryos normalised to non-injected (NI) controls. In each case, both PBS and *E. coli* injection significantly increased antimicrobial gene expression, with *E. coli* inducing the greatest level of gene expression. However, the induction of antimicrobial genes via PBS injection inferred that damage may also play a role in the induction of systemic immune response genes, particularly those where the fold increase in expression was large (*Cec,* B). * Indicates $p \leq 0.05$, ** indicates $p \leq 0.01$. All other changes should be considered as non-significant. Error bars represent standard deviation.


A
Dpt
Fold difference in expression (AU)
50.0
40.0
30.0
20.0
10.0
0.0
**
**
*
NI
PBS
DH5α E. coli
B
CecA1
Fold difference in expression (AU)
60.0
40.0
20.0
0.0
**
*
NI
PBS
DH5α E. coli

3.2.2 *Drc-GFP* expression is localized in tracheal and epidermal tissues within the Stage 15 Drosophila embryo upon bacterial infection

Having observed that Stage 15 *Drosophila* embryos were able to mount a robust AMP response to a simple bacterial infection, it was necessary to determine the precise localization of this response. Using confocal microscopy permitted an initial assessment of the type of cells in which *Drc-GFP* was expressed upon *Ecc15* infection (OD=1; Figure 3.7A). In particular, z-stacks acquired of infected embryos at 6 hours post infection indicated that these cells appeared to be highly present in the anterior region of the embryo, the location where the bacterial stimulus was initially administered. In this region, intense bands of GFP-positive cells forming an almost segmental pattern were noted although no specific pattern of individual *Drc-GFP* expressing cells was apparent (Figure 3.7A), concordant with previous results (Figure 3.5). To elucidate the precise localisation site of *Drc* expression, and hence to identify the type of cell expressing the *Drc-GFP* construct, Stage 15 *Drc-GFP* embryos expressing moesin-cherry (mCherry) in the epithelia, under the control of the *E22c* promoter using the Gal4 UAS system, were infected with *Ecc15* (OD=1). Z-stack images of the resulting response were obtained, as can be seen in Figure 3.7B and C. Co-localisation of the *Drc-GFP* and *E22c>mCherry* signals was not immediately apparent (Figure 3.7B), although both signals appeared to co-exist within the same focal planes. However, upon close examination via orthogonal views (Figure 3.7C), it is clear that the *Drc-GFP* signal originated from cells within the epidermal layer of the Stage 15 embryo. This would suggest that the cells expressing *Drc-GFP* within the Stage 15 embryos at 6 hours post infection are of epidermal origin.

Further to this, immunostaining was performed to determine other principle locations of *Drc* expression within the Stage 15 *Drc-GFP* embryo at earlier time-points post infection (Figure 3.8). *Drc-GFP* embryos were infected with *Ecc15* (OD=1) and were fixed at 3 hours post injection. Subsequently, embryos were immunostained for GFP, as an inference of *Drc* expression. In light of the finding that the Stage 15 embryo was able to induce AMP expression upon damage alone (Figure 3.6), Stage 15 embryos injected with

sterile endotoxin-free PBS were also prepared in the same manner; to investigate the localization of *Drc-GFP* expression upon damage stimuli. Non-injected (NI) *Drc-GFP* embryos were also immunostained in the same manner as an internal control.

Results yielded the interesting observation that *Drc-GFP* was also expressed within the embryonic tracheal network at 3 hours post injection with both sterile endotoxin-free PBS and *Ecc15*, whilst NI embryos lacked any *Drc-GFP* expression (Figure 3.8A). The response itself appeared extensive, with *Drc-GFP* expression noted in at least primary and secondary tracheal branches along the entire length of the embryo, despite the initial infection being localized exclusively in the anterior. This would imply that both damage and bacterial infection are able to stimulate an extensive AMP response within tracheal cells. Confirmation of the identity of these cells was obtained via the co-immunostaining of *Drc-GFP* and the trachea luminal maker 2A12 (Figure 3.8B), where a close co-localisation of *Drc-GFP* and 2A12 signal was observed. Whilst 2A12 staining provided visualization of the tracheal lumen, *Drc-GFP* expression itself appeared to the localized to the tracheal epithelium (Figure 3.8B), with Drc-GFP expressing cells apparently surrounding the tracheal lumen.

Consequently, the expression of *Drc-GFP* at 6 hours post infection with *Ecc15* appeared to be localized to cells embedded in the embryonic epidermal tissue. This response was shown to be extensive and have some potential segmental bias, although no clear pattern between individual expressing cells was apparent. This epidermal response was also shown to be supplemented by an earlier *Drc-GFP* response at 3 hours post infection, localized to the tracheal network, which could be induced either by damage alone or by infection with *Ecc15*.

Figure 3.7: Stage 15 *Drc-GFP* expression is localized to the epidermis at 6 hours post infection

(**A**) Z-stack imaging of the anterior of an infected Stage 15 embryo 6 hours post infection with *Ecc15* (OD=1; n=3 where ≥25 embryos were employed per replicate). *Drc-GFP* expression is extensive, and almost segmental in nature although no clear pattern is apparent. (**B-C**) Z-stack images of *Drc-GFP/e22c>mCherry* embryos infected with *Ecc15* (OD=1, n=3 where ≥25 embryos were employed per replicate). Co-localisation of *Drc-GFP* (green) and *e22c>mCherry* (red) signals was not immediately apparent (**B**), although orthogonal projections through *Drc-GFP/e22c>mCherry* embryos shows that *Drc-GFP* expressing cells are embedded within the epidermis of infected embryos (**C**), suggesting that these cells are epidermal in origin. Scale bars represent 20μm.

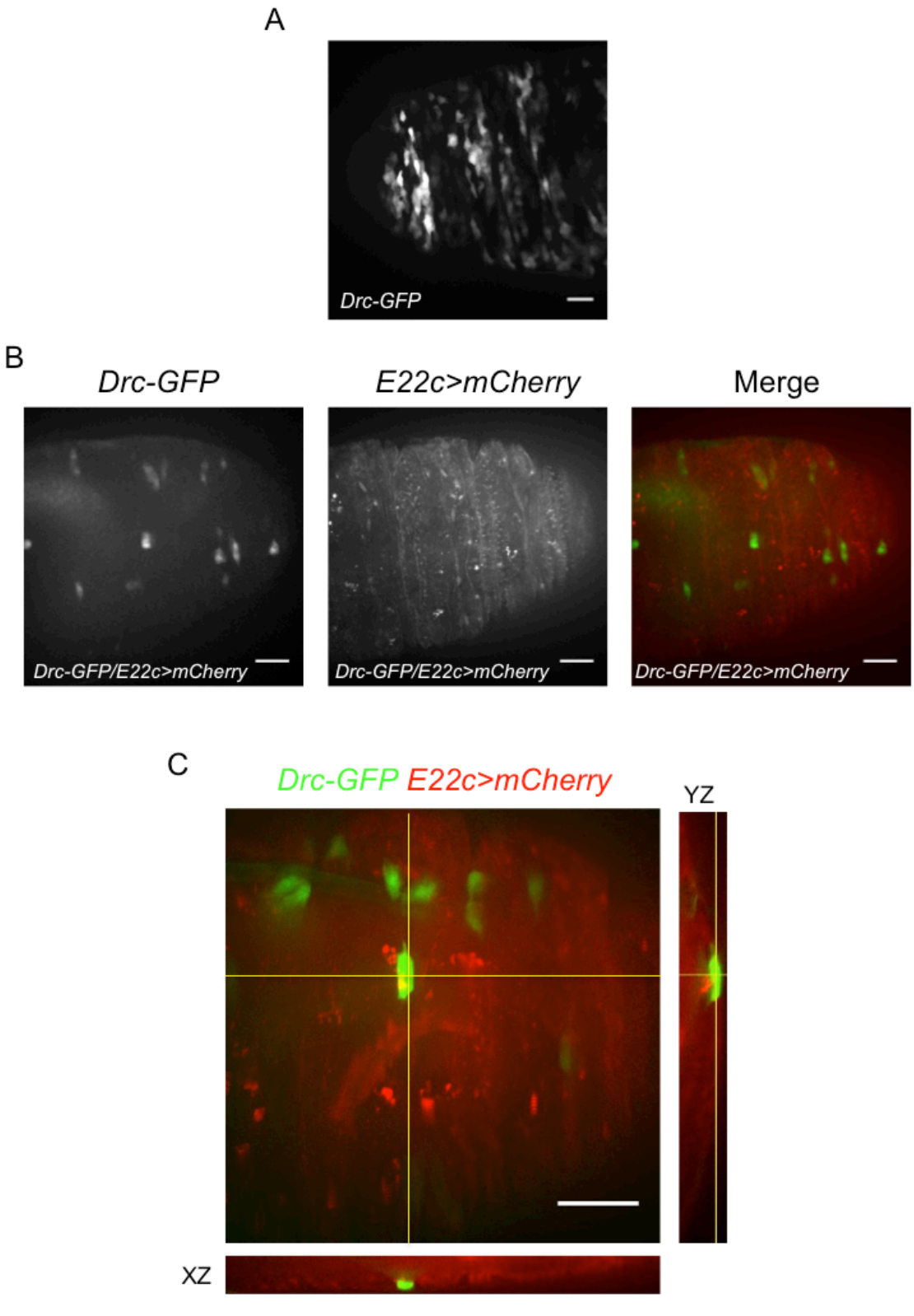
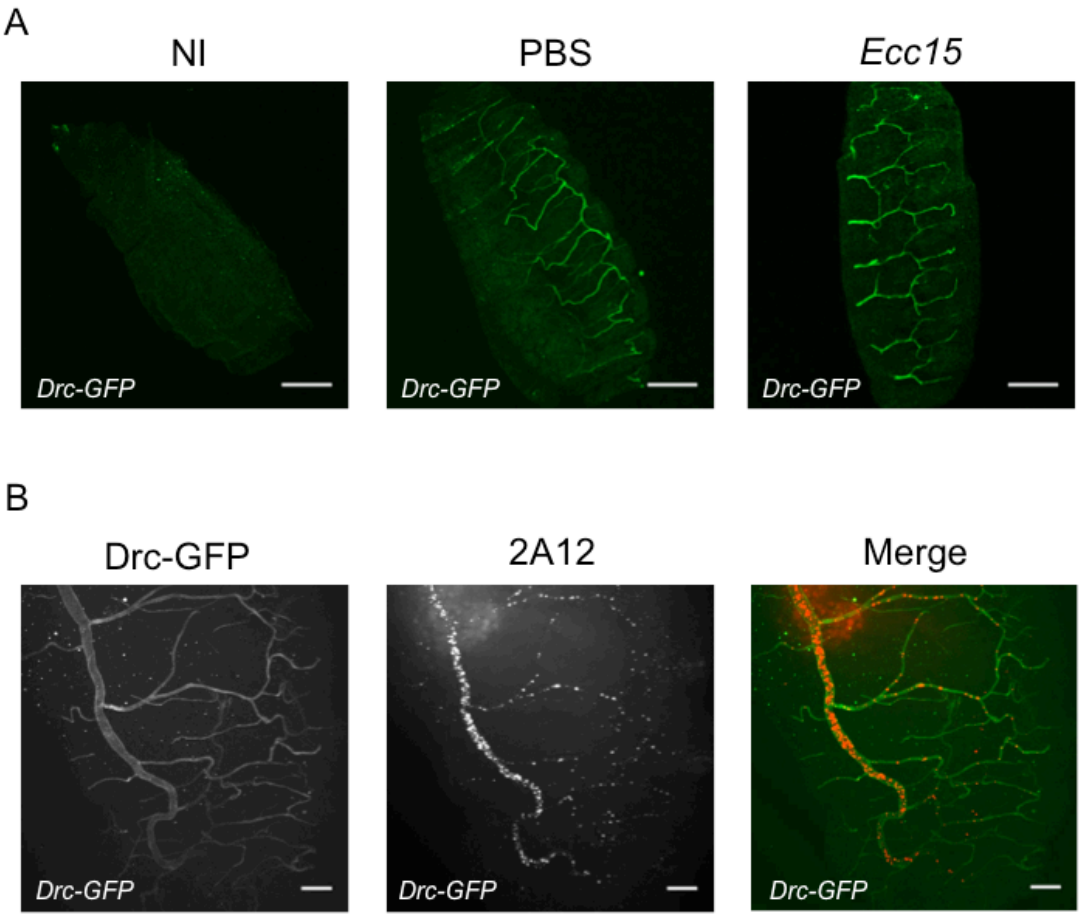

A
Drc-GFP
B
Drc-GFP
E22c>mCherry
Merge
Drc-GFP/E22c>mCherry
Drc-GFP/E22c>mCherry
Drc-GFP/E22c>mCherry
C
Drc-GFP E22c>mCherry
YZ
XZ

Figure 3.8: Damage and *Ecc15* Infection induce *Drc-GFP* expression within the tracheal network

(**A**) Immunostaining for *Drc-GFP* in Stage 15 embryos at 3 hours post injection with sterile endotoxin-free PBS or *Ecc15* (OD=1) demonstrated expression of the construct in the tracheal network. Non-injected (NI) embryos exhibited a lack of *Drc-GFP* expression in comparison, indicating that the expression observed was a result of damage or infection. Scale bars represent 50μm. (**B**) The localization of this expression was confirmed by co-immunostaining for *Drc-GFP* (green) and the tracheal luminal marker 2A12 (red). Scale bars represent 20μm.


A
NI
PBS
Ecc15
Drc-GFP
Drc-GFP
Drc-GFP
B
Drc-GFP
2A12
Merge
Drc-GFP
Drc-GFP
Drc-GFP

3.2.3. Stage 15 embryos are able to differentiate between different types of infection

Having established that the Stage 15 embryo was able to mediate a robust systemic AMP response to bacterial challenge, it was assessed whether embryos at this stage of embryogenesis were also able to distinguish between different types of bacterial pathogen, in a comparable manner to adult fly and larval infection models. WT embryos were microinjected either with sterile endotoxin-free PBS, *Ecc15* (OD=1) or *M. luteus* (OD=1) and expression of AMP genes in response to these treatments at 2 hours post injection assessed by RT-qPCR (Figure 3.9).

Whilst those AMP genes typically induced by Gram-negative infection, such as *AttA*, *CecA1, Dpt* and *Drc* (Wicker *et al.,* 1990; Dimarcq *et al.,* 1993; Asling *et al.,* 1995), were significantly induced by Gram-negative *Ecc15* treatment, *Drosomycin* (*Drs*), a Gram-positive bacterial and fungal AMP gene (Fehlbaum *et al.,* 1994; Bischoff *et al.,* 2004), was preferentially induced by *M. luteus* injection (Figure 3.9A-B). Therefore, the basic specificity of AMP gene induction that is mediated in response to Gram-negative and Gram-positive infection in the *Drosophila* adult and larval systems is also functional in the Stage 15 embryos. However, not all data is indicative of adherence to the basic AMP classification determined in other *Drosophila* models. For instance, *M. luteus* infection did not induce any significant expression of *Def,* an AMP which has been shown to be typically induced upon Gram-positive infections in *Drosophila* (Figure 3.9B). Moreover, *Ecc15* infection significantly induced expression of both *Def* and *Mtk*, AMPs expressed in response to Gram-positive bacterial and fungal infections respectively (Figure 3.9B)

To ascertain whether this differential response in the Stage 15 embryo was mediated via canonical IMD and Toll signaling pathways, *Dpt, Drc* and *Drs* expression in response to *Ecc15* and *M. luteus* injection was assessed via RT-qPCR at 2 hours post injection in *relish*^{e20} (*rel*^{e20}) and *modular serine protease*^{1} (*modSP*^{1}) mutant embryos, whereby IMD and Toll signaling was respectively disrupted (Figure 3.10A-C). *Dpt* and *Drs* expression in response

to sterile PBS injection treatment was also assessed in WT and mutant embryos as an internal control. Both *Drc* and *Dpt* expression in response to *Ecc15* infection was completely abolished in rel^{e20} embryos at 2 hours post injection (Figure 3.10A-B), with the relative expression of both AMPs significantly diminished in comparison to WT levels ($p \leq 0.01$ and $p \leq 0.001$ respectively). In fact, expression levels of *Dpt* and *Drc* upon *Ecc15* infection were not significantly different to those noted upon PBS injection alone (Figure 3.10A-B). Moreover, this effect on *Dpt* expression is specific to *Ecc15* infection, as injection with sterile endotoxin-free PBS did not significantly induce *Drc* or *Dpt* expression in WT or mutant embryos (Figure 3.10A-B). Similarly, $modSP^{1}$ mutant embryos were unable to induce *Drs* expression to WT levels, showing a highly significant reduction in *Drs* expression upon *M. luteus* infection ($p \leq 0.001$; Figure 3.9C). Thus, *Drs* expression within the embryo is a direct result of Toll pathway activation.

Moreover, the absence of IMD or Toll signaling was also shown to impact on Stage 15 embryo viability post infection. The survival of rel^{e20}, $modSP^{1}$ and $persephone^{1}$ $(psh^{1});;modSP^{1}$ double mutant embryos 24 hours after injection with either sterile endotoxin-free PBS, *DH5α E. coli* (OD=1), *M. luteus* (OD=1), *Erwinia carotovora carotovora 15* (*Ecc15*; OD=0.1), or proteases from *Aspergillus oryzae* (1:5000 dilution) was monitored and compared to survival levels for WT control embryos (Figure 3.10D). Neither the damage inflicted via the microinjection of sterile endotoxin-free PBS nor infection with *DH5α E. coli* significantly affected the survival of WT or immune mutant embryos post injection. Conversely, injection with *Ecc15* selectively and significantly reduced the viability of rel^{e20} mutants compared to WT controls (17% ± 9.64, $p \leq 0.01$), whereas *M. luteus* injection significantly reduced the survival of both $modSP^{1}$ and $psh^{1};;modSP^{1}$ mutants compared to WT viability levels (47% ± 8.21, $p \leq 0.001$ and 34% ± 0.707, $p \leq 0.001$ respectively), confirming that IMD and Toll signaling are required in the Stage 15 embryos for effective Gram-negative and Gram-positive infection resolution respectively. Interestingly, $modSP^{1}$ and $psh^{1};;modSP^{1}$ embryo viability was also decreased upon *Ecc15* infection compared to that of WT embryos, although this effect was not statistically

significant. *modSP*^{1} mutant viability was also significantly reduced upon injection of *A. oryzae* proteases (45% ± 2.65, p≤0.05). However, whilst *psh*^{1}*;;modSP*^{1} survival was decreased by injection with *A. oryzae* proteases (50%), this reduction was determined to not be statistically significant in comparison to levels of WT embryo mortality. Therefore, whilst the majority of data suggests that the systemic immune response is mediated via the IMD and Toll pathways in the embryo, in concordance with adult and larval models, this exception would indicate that not all mechanisms operate in the same manner in the embryo.

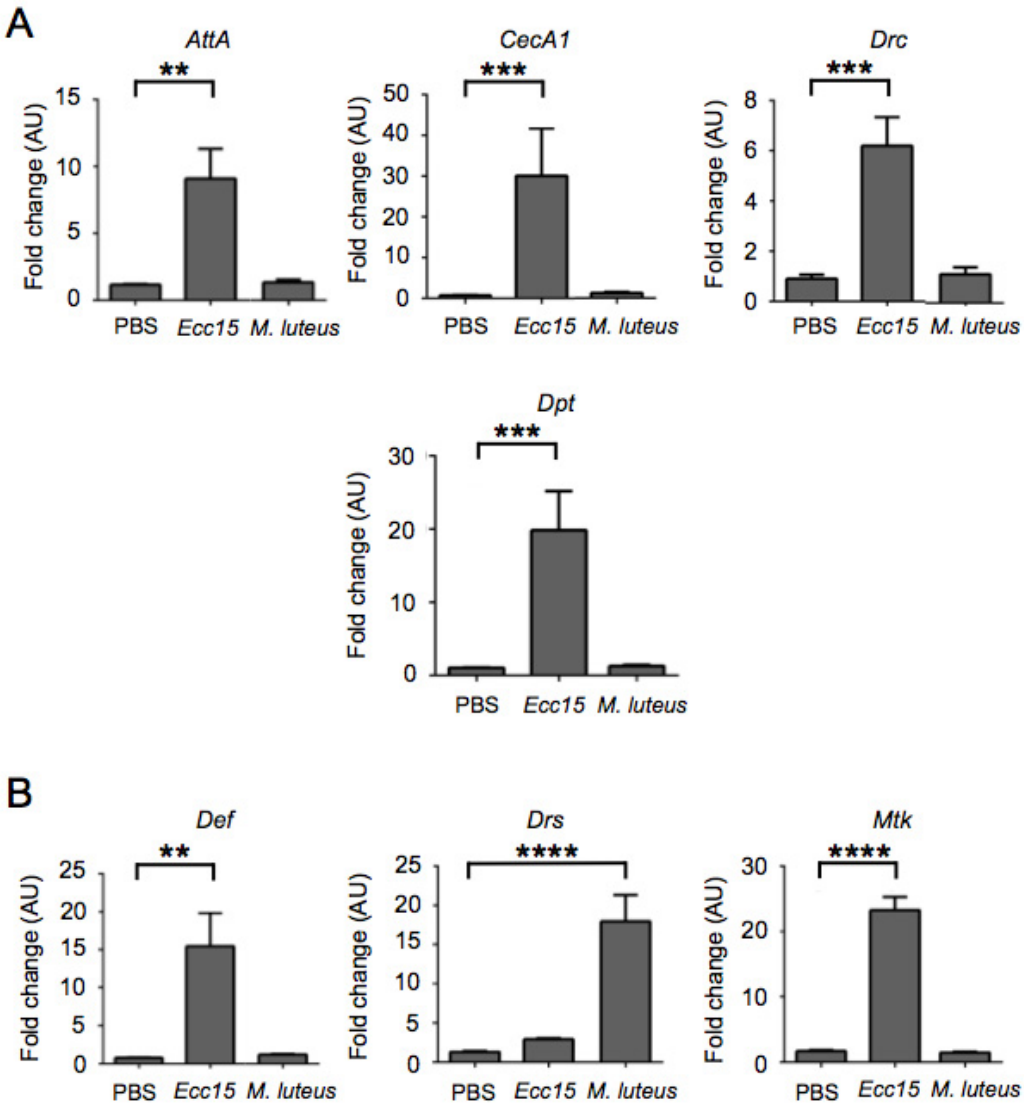
Nevertheless, the ability of the embryo to differentiate between different invading pathogens should not be considered as a static process. We subsequently sought to determine if Stage 15 embryos were able to differentiate between different Gram-negative bacterial species. WT embryos were infected either with sterile endotoxin-free PBS, *Ecc15* (OD=1) or *Mg1655 E. coli* (OD=1) and their subsequent expression of AMP genes 2 hours post injection was elucidated by RT-qPCR. Relative fold difference in expression was then calculated by normalizing to PBS-injected levels (Figure 3.11). Both *Mg1655 E. coli* and *Ecc15* stimulated significant increases in Gram-negative AMP expression relative to PBS-injected levels (Figure 3.11A-B, D-E). Further to this, the level of expression for each gene was calculated as a proportional of the total AMP response, hence providing information about the cocktail of AMP genes expressed upon each infection. The induction of the majority of Gram-negative AMPs was comparable between the two species, as in the cases of *AttA, CecA1* and *Drc,* where no significant difference was noted between expression levels in *Mg1655 E. coli* and *Ecc15* treated embryos. The one exception is the expression of *Dpt* which, whilst significantly increased upon infection with both species, was more highly induced upon *Ecc15* infection. This may suggest a preference for the induction of *Dpt* to combat *Ecc15* invasion. Intriguingly, this trend was also noted with AMPs of other classes, which are not typically renowned for expression upon Gram-negative bacterial infection. For instance, *Mg1655 E. coli* and *Ecc15* both induced significant upregulation of *Def* expression and *Mtk* expression (Figure 3.11C

and G), which is usually observed in response Gram-positive and fungal infection respectively; in both cases, *Ecc15* provoked a more intense response than *Mg1655 E. coli*. No significant upregulation of *Drs* was noted upon infection with either species (Figure 3.11F). Nevertheless, when taking into account the proportion that individual AMPs contribute to the overall response (Figure 3.11H), it is clear that *Mg1655 E. coli* and *Ecc15* infection both induce a similar cocktail of AMP gene expression. Both Gram-negative species stimulate a preferentially high level of *CecA1* expression, with other AMPs such as *AttA, Def* and *Dpt* induced to similar degrees. Therefore, even though in many cases *Ecc15* stimulated higher levels of AMP gene expression than *Mg1655 E. coli*, the contribution of each AMP to the total response is relatively similar for each bacterium. However, the embryo's responses to these different species are not identical. There appears to be a preference for *Mg1655 E. coli* to stimulate *CecA1, AttA* and *Drs*. On the other hand, this data confirms that *Ecc15* stimulates a greater response of *Mtk* and *Drc* than its *Mg1655 E. coli* counterpart.

Collectively, these results allow us to draw the conclusion that WT Stage 15 *Drosophila* embryos are able to mount a specific AMP response to bacterial infection, that is not only able to distinguish between Gram-negative and Gram-positive infections but also is tailored to the individual species of invading pathogen, and that this is mediated without any significant consequences on subsequent WT embryo survival. Moreover, these responses are mediated via the well-characterised canonical IMD and Toll signaling pathways that have been shown to play a crucial role in the systemic responses of *Drosophila* adults and larvae.

Figure 3.9: Stage 15 *Drosophila* embryos are able effectively distinguish between different types of infection

(**A-B**) Bar charts displaying relative levels of AMP gene expression in Stage 15 embryos (n=3, with ≥200 embryos per experiment) upon treatment with either *Ecc15* (OD=1) or *M. luteus* (OD=1). Whilst *Ecc15* treatment preferentially stimulated Gram-negative AMP expression (**A**), *M. luteus* infection only significantly induced *Drs* expression (**B**). These results confirm that the embryo is able to differentiate between different types of infection. Surprisingly, *Ecc15* treatment also significantly induced *Def* and *Mtk* expression, which are classified as Gram-positive and fungal AMPs respectively, whilst *M. luteus* did not significantly induce expression of either of the aforementioned AMPs (**B**). * Indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$. All other changes should be considered as not statistically significant. Error bars represent standard deviation.


A
AttA
**
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15
10
5
0
PBS
Ecc15
M. luteus
CecA1
***
Fold change (AU)
50
40
30
20
10
0
PBS
Ecc15
M. luteus
Drc
***
Fold change (AU)
8
6
4
2
0
PBS
Ecc15
M. luteus
Dpt
***
Fold change (AU)
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20
10
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PBS
Ecc15
M. luteus
B
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**
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25
20
15
10
5
0
PBS
Ecc15
M. luteus
Drs
****
Fold change (AU)
25
20
15
10
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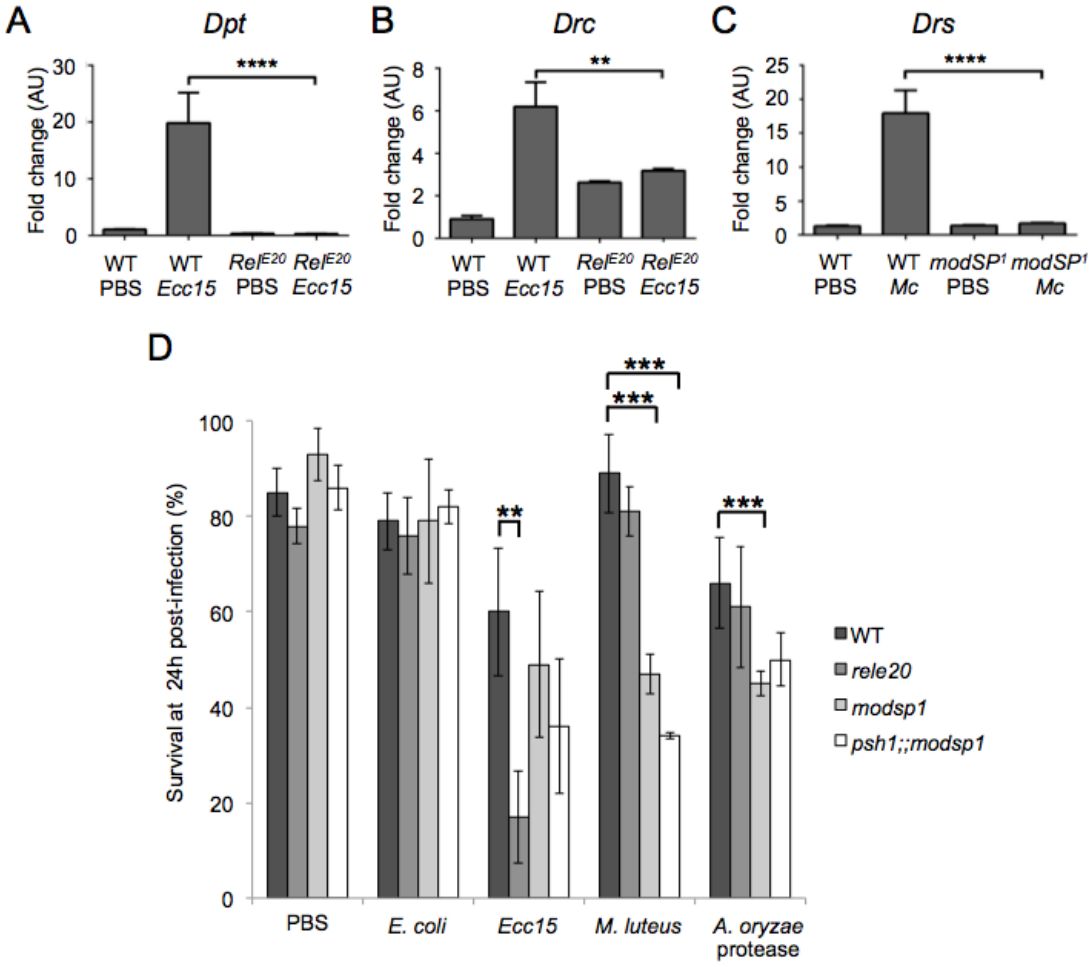
0
PBS
Ecc15
M. luteus
Mtk
****
Fold change (AU)
30
20
10
0
PBS
Ecc15
M. luteus

Figure 3.10: AMP gene induction is mediated via canonical immune signaling in the Stage 15 *Drosophila* embryo.

(A-C) Bar charts showing the fold change in expression levels of different AMP genes in WT, *rel*^{e20} and *modSP*^{1} embryos upon Gram-negative and Gram-positive infection, normalised to expression levels observed in PBS injected embryos. *Dpt* expression was completely and significantly inhibited in *rel*^{e20} embryos upon *Ecc15* infection, in comparison to WT controls (**A;** One-way ANOVA, N= where each replicate consisted of ≥200 embryos). Moreover, PBS injection significantly induced *Dpt* expression. A similar trend was noted for *Drc* expression when *rel*^{e20} and WT expression upon *Ecc15* infection (**B**). Conversely, *Drs* expression in modSP1 embryos was highly significantly downregulation compared to levels observed in WT embryos (**C**). (**D**) Bar graph showing viability of Stage 15 wild-type control embryos (WT) and Stage 15 *rel*^{e20}, *modsp*^{1} and *psh*^{1}*;;modSP*^{1} mutant embryos injected with PBS, *E. coli, Ecc15, M. luteus* or proteases from the fungus *A. oryzae*. Whilst injection of PBS or *DH5α E. coli* did not significantly impact the viability of WT or mutant embryos, infection with *Ecc15* selectively diminished the survival of *rel*^{e20} mutants 24h post infection. Similarly, injection with *M. luteus* specifically decreased the viability of *modSP*^{1} and *psh*^{1}*;;modSP*^{1} mutant embryos, although injection with *A. oryzae* proteases (1:2000) only significantly reduced the survival of *modSP*^{1} mutants. Taken together, these results infer that AMP induction in the embryonic systemic immune response is induced via the canonical IMD and Toll signaling pathways.

* Indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$. All other changes should be considered as not statistically significant. Error bars represent standard deviation.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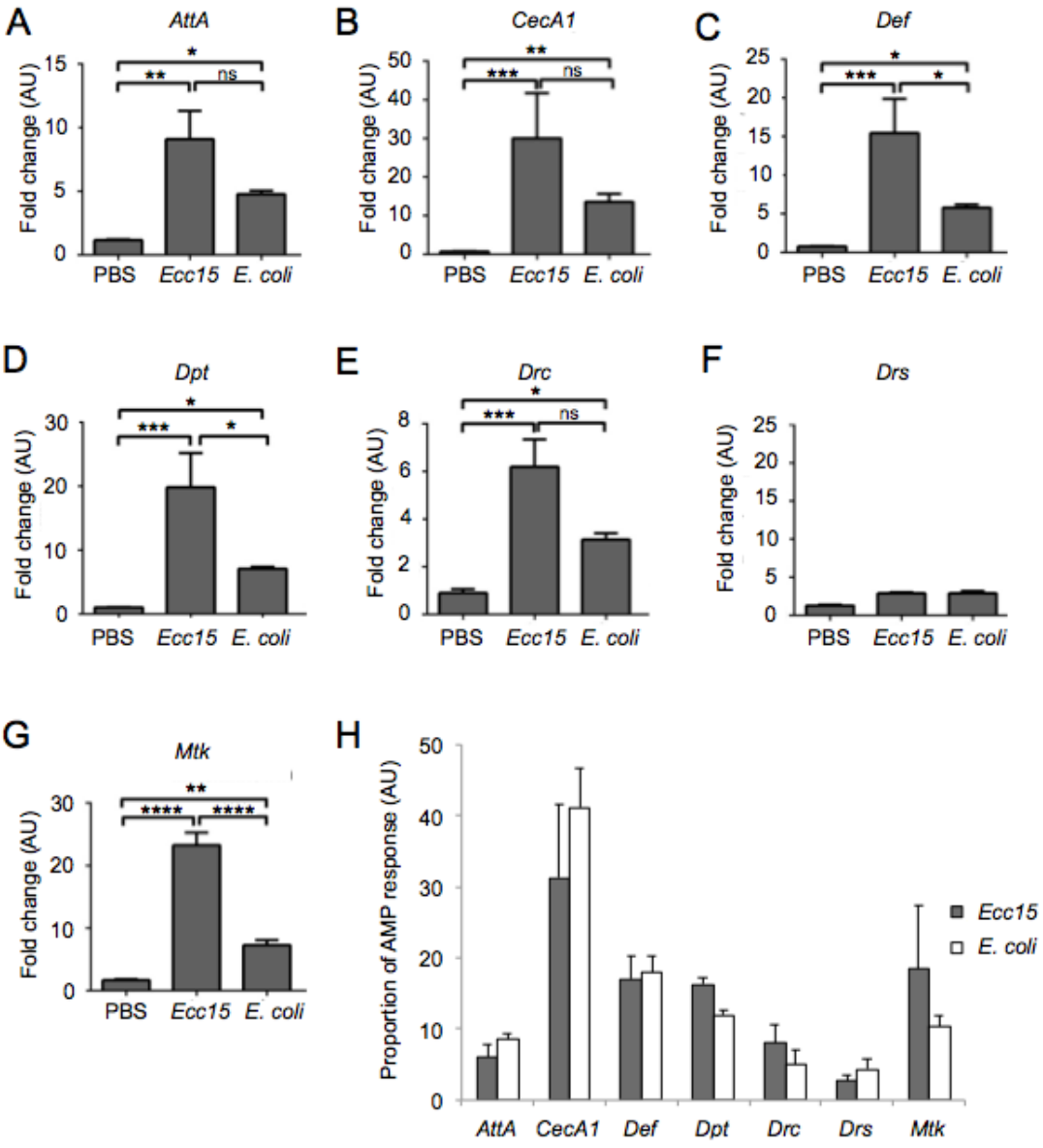
20
10
0
****
WT PBS
WT Ecc15
RelE20 PBS
RelE20 Ecc15
B
Drc
Fold change (AU)
8
6
4
2
0
**
WT PBS
WT Ecc15
RelE20 PBS
RelE20 Ecc15
C
Drs
Fold change (AU)
25
20
15
10
5
0
****
WT PBS
WT Mc
modSP1 PBS
modSP1 Mc
D
Survival at 24h post-infection (%)
100
80
60
40
20
0
**
***
***
***
PBS
E. coli
Ecc15
M. luteus
A. oryzae protease
WT
rele20
modsp1
psh1;;modsp1

Figure 3.11: Stage 15 embryos are able to adapt the AMP response to different Gram-negative infections

(**A-G**) Bar charts showing relative levels of AMP gene expression in sterile endotoxin-free PBS, *Ecc15* and *Mg1655 E. coli* injected Stage 15 embryos. Both *Ecc15* and *Mg1655 E. coli* significantly induced expression of *AttA*, *CecA1, Def, Dpt, Drc* and *Mtk* above PBS injected relative levels (**A-E, G**). Neither infection stimulated significant *Drs* expression above PBS-injected control levels (**F**). *Ecc15* provoked a significantly greater induction of *Def, Dpt* and *Mtk* expression than *Mg1655* infection (**C, D, G**). (**H**) When considering individual AMP gene expression as a proportion of the total response, both bacteria stimulated a high degree of *CecA1* expression and moderate levels of *Dpt* and *Def* expression. However, *Ecc15* was shown to stimulate a higher proportion of *Mtk* and *Dpt* expression than *Mg1655 E. coli*. Conversely, *Mg1655* stimulated a high proportion of *CecA1, Drs* and *Def* expression. * Indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$. All other changes should be considered as not statistically significant. Error bars represent standard deviation.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AttA
Fold change (AU)
PBS
Ecc15
E. coli
B
CecA1
C
Def
D
Dpt
E
Drc
F
Drs
G
Mtk
H
Proportion of AMP response (AU)
AttA
CecA1
Def
Dpt
Drc
Drs
Mtk
Ecc15
E. coli

3.2.5 *Drosophila* embryos are able to mount a robust cellular response to bacterial infection that depends on organization of the actin cytoskeleton

To determine if the cellular branch of the immune response was intact and able to mediate the clearance of bacterial species within the *Drosophila* embryo, Stage 15 embryos containing GFP-labelled plasmatocytes were injected with RFP-expressing *E. coli*, to determine if plasmatocytes could effectively phagocytose bacteria at this stage of development. Moreover, it was also investigated whether components of the actin cytoskeleton which had been previously demonstrated to play key roles in phagocytosis were required for embryonic plasmatoocyte clearance of pathogen. In this case, the precise role of SCAR, a component of the Arp2/3 complex which regulates actin nucleation (Machesky *et al.,* 1999; Welch and Mullins, 2002; Zallen *et al.,* 2002), in embryonic phagocytosis was investigated.

Live imaging at 1 hour post injection revealed that Stage 15 embryonic hemocytes were able to effectively phagocytose injected RFP-*E. coli*, with a mean value 40% of total bacteria injected at this time-point (Figure 3.12A), hence demonstrating that plasmatocytes at Stage 15 of embryogenesis are effectively able to recognize and engulf bacteria. However, $SCAR^{\Delta 37}$ mutant plasmatocytes phagocytosed RFP-*E. coli* at significantly reduced levels compared to WT, with only 15% of total RFP-*E. coli* successfully phagocytosed at 1 hour post infection (Figure 3.12A). Furthermore, non-phagocytosed RFP-*E. coli* were often observed adhered to the surface of $SCAR^{\Delta 37}$ mutant plasmatocytes, which is suggestive of an inability to phagocytose as opposed to a defect with recognition.

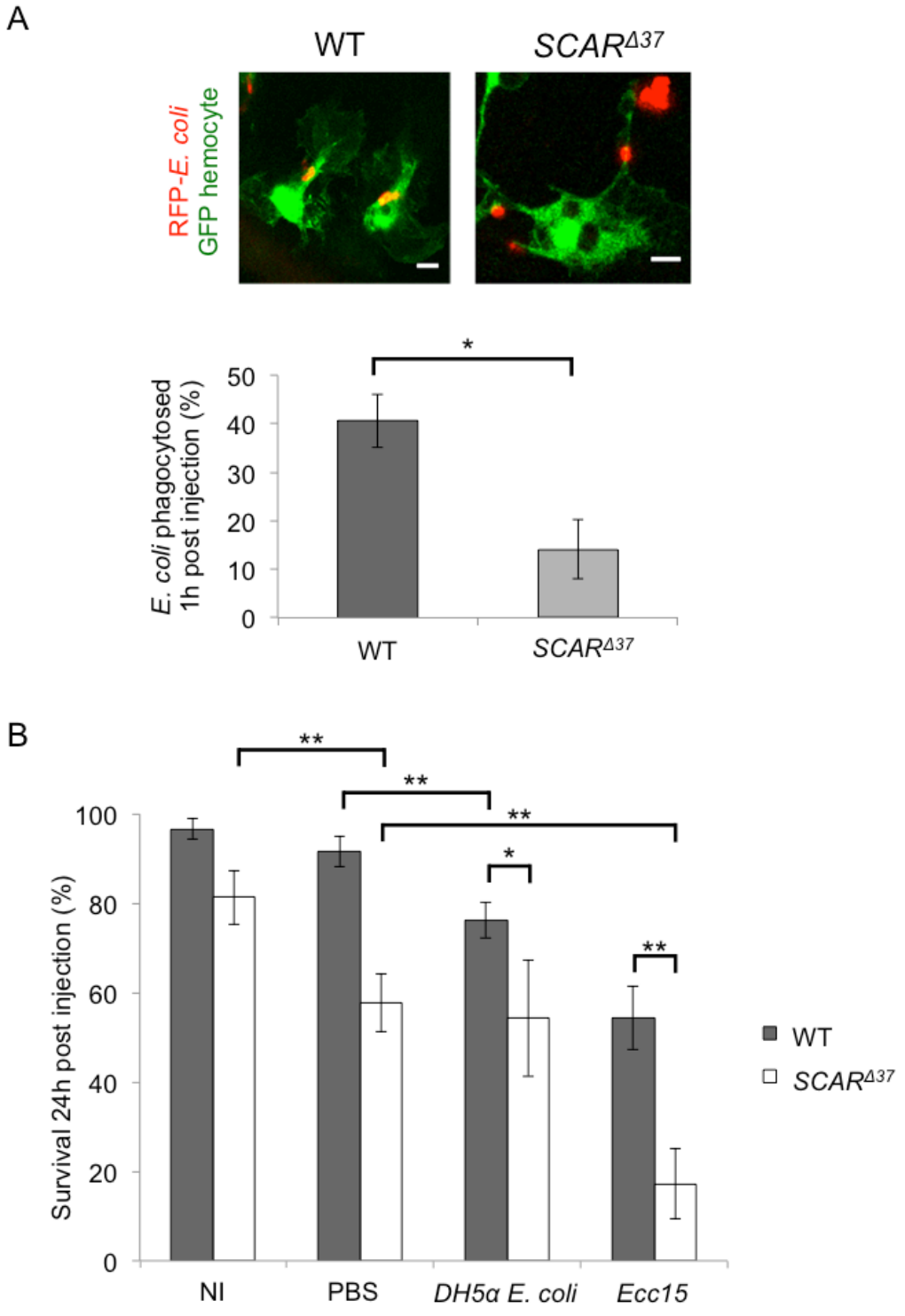
To assess the resulting biological cost of defective clearance, the viability of WT and $SCAR^{\Delta 37}$ embryos was monitored for 24 hours after infection with either *DH5α E. coli* or *Ecc15* (Figure 3.12B). Infection with *Ecc15* resulted in a decrease in viability in both WT and $SCAR^{\Delta 37}$ mutant embryos beyond that observed for PBS injected control embryos. Crucially, this *Ecc15*-associated mortality was significantly enhanced in $SCAR^{\Delta 37}$ mutants, inferring that a failure

in SCAR-dependent phagocytosis substantially compromised the embryonic innate immune response as a whole. Interestingly, comparison of non-infected (NI) and PBS treated $SCAR^{\Delta 37}$ mutants identified a significant reduction in viability in response to this treatment (Figure 3.12B), suggesting that SCAR may also play a role in a response to damage or repair processes.

Another interesting feature of the data is that infection with *DH5α E. coli* did not further or significantly reduce the levels of viability of $SCAR^{\Delta 37}$ embryos from that observed upon PBS injection (Figure 3.12B). This is in contrast to the WT response, whereby infection with *DH5α E. coli* induces a significant decrease in embryo viability ($p \leq 0.01$, Figure 3.12B). This may suggest that the lack of phagocytosis in a $SCAR^{\Delta 37}$ mutant background confers some biological benefit. Therefore, the data implies that, in addition to the Stage 15 embryo's systemic capabilities, the cellular branch of the immune response is also active and able to mediate the effective clearance of infection at this stage of development. This process requires correct organization of the actin cytoskeleton in primary phagocytes, as SCAR mutant plasmatocytes are unable to effectively clear infective agents. This inability to effectively phagocytose invading pathogens ultimately leads to increased mortality, inferring the crucial role of phagocytosis in the embryonic immune response.

Figure 3.12: SCAR is required for mediating successful phagocytosis in the Stage 15 embryo

(**A**) Wild-type (WT) or *SCAR*$^{\Delta 37}$ Stage 15 embryos with hemocytes labeled with GFP (green) were injected with RFP-labelled *E. coli* (red) and imaged at 1 hour post infection. This revealed a defect in *SCAR*$^{\Delta 37}$ mutant phagocytosis compared to WT controls. A significant reduction was noted in the percentage of RFP-*E.coli* phagocytosed in SCAR mutants ($p \leq 0.01$, Mann-Whitney U-test, $n \geq 4$ per genotype). Scale bars represent 5μm. (**B**) Bar graph showing mean survival of WT and *SCAR*$^{\Delta 37}$ non-injected controls (NI) and PBS, *DH5α E. coli* or *Ecc15* injected embryos. Injection with PBS, *DH5α E. coli* or *Ecc15* significantly decreased the viability of Stage 15 SCAR mutant embryos 24 hours post infection compared to WT counterparts ($p \leq 0.01$, $p \leq 0.05$, and $p \leq 0.01$, respectively, Unpaired T-Test; n=3, where ≥100 embryos were employed per experiment). * Indicates $p \leq 0.05$, ** indicates $p \leq 0.01$. Error bars represent standard deviation.


A
WT
SCAR$^{\Delta 37}$
RFP-E. coli
GFP hemocyte
E. coli phagocytosed 1h post injection (%)
50
40
30
20
10
0
*
WT
SCAR$^{\Delta 37}$
B
Survival 24h post injection (%)
100
80
60
40
20
0
**
**
**
*
**
NI
PBS
DH5α E. coli
Ecc15
WT
SCAR$^{\Delta 37}$

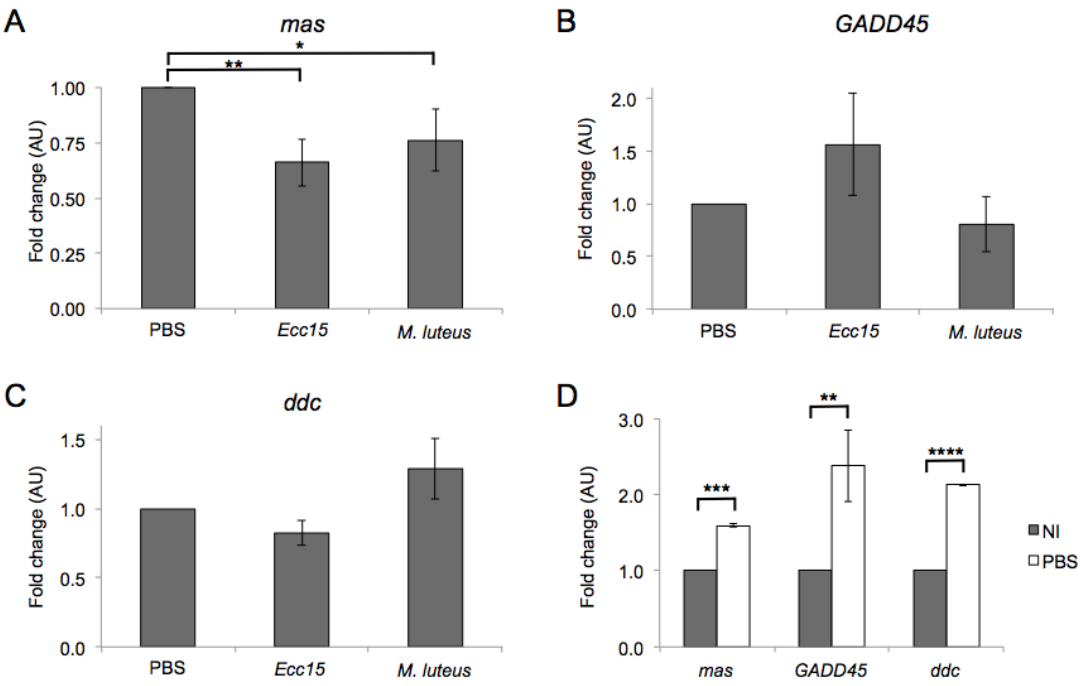
3.2.6 Stage 15 embryos are able to respond to bacterial infection using a diverse range of immune mechanisms

Following from the notion that multiple immune mechanisms may be intact within the embryo and able to facilitate an effective response to infection, RT-qPCR was used to screen for changes in expression levels of immune genes implicated in more diverse roles in the *Drosophila* immune responses to bacterial infection and damage. These included the *dopa decarboxylase* (*ddc*), *masquerade* (*mas*) and *Growth Arrest and DNA Damage-inducible 45* (*GADD45*) genes, which play roles in melanisation, regulation of serine proteases upon infection and damage-induced stress responses respectively (Irving *et al.,* 2001; Stramer *et al.,* 2007; Tang, 2009). Relative expression levels for each gene upon infection for damage were normalized to non-infected or PBS-injected levels as appropriate.

Expression of some candidate genes, as in the case of *mas,* was observed to be significantly downregulated upon infection with both Gram-negative and Gram-positive microbes, such as *Ecc15* and *M. luteus* (Figure 3.13A; $p \leq 0.01$ and $p \leq 0.05$ respectively), suggesting a role for mas in the Stage 15 innate immune response. However, whilst other candidate genes demonstrated increases in expression on specific types of infection, such as the 1.56-fold induction of *GADD45* upon *Ecc15* infection (Figure 3.13B), these changes were observed to be non-significant. Similarly, *ddc* expression was apparently downregulated upon *Ecc15* infection and increased upon injection with *M. luteus*, but in a non-significant manner (Figure 3.13C). Conversely, all relative expression levels of all three aforementioned candidate genes were highly and significantly increased upon PBS injection, when compared to relative levels in NI controls (Figure 3.13D), inferring a role for these genes in the embryonic damage response and potential cross-talk between the *Drosophila* immune system and repair mechanisms. Therefore, infection of Stage 15 embryos with Gram-negative and Gram-positive bacteria is able to induce a wide range of transcriptional responses that are not restricted to AMP gene modulation, and thus is suggestive of a more complete and functional embryonic immune system.

Figure 3.13: A diverse range of immune genes are activated within the Stage 15 embryo upon bacterial infection and damage

RT-qPCR was used to determine if the activity of other genes implicated in the *Drosophila* immune response was modulated upon bacterial infection (**A-C**) or upon damage (**D**) in Stage 15 embryos, with relative levels of gene expression normalized to control PBS-injected or non-infected (NI) levels where appropriate. *Masquerade* (*mas*) expression was demonstrated to be significantly reduced upon infection with *Ecc15* or *M. luteus* (**A**; One way ANOVA, n=3 with ≥ 200 embryos per experiment, p≤0.01 and p≤0.05 respectively). The expression of *Growth Arrest and DNA Damage-inducible 45* (*GADD45*) was increased upon *Ecc15* infection, although this change was not significant (**B**). Likewise, *dopa decarboxylase* (*ddc*) expression was increased upon *M. luteus* and decreased upon *Ecc15* infections respectively in a non-significant manner (**C**). However, the expression of all three immune genes was demonstrated to be highly significantly induced by PBS injection, indicating that damage may activate transcription of these candidate genes (**D**; One-way ANOVA, n=3 with ≥ 200 embryos per replicate). * Indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001, **** indicates p ≤ 0.0001. Error bars indicate standard deviation.


A
mas
*
**
1.00
0.75
0.50
0.25
0.00
Fold change (AU)
PBS
Ecc15
M. luteus
B
GADD45
2.0
1.5
1.0
0.5
0.0
Fold change (AU)
PBS
Ecc15
M. luteus
C
ddc
1.5
1.0
0.5
0.0
Fold change (AU)
PBS
Ecc15
M. luteus
D
3.0
2.0
1.0
0.0
Fold change (AU)
***
**
****
NI
PBS
mas
GADD45
ddc

3.2.7 Stage 11 embryos show reduced immune competency compared to Stage 15 embryos

To assess whether embryos at other stages of embryogenesis displayed the same level of immune competence demonstrated at Stage 15, the AMP response of Stage 11 embryos to Gram-negative bacterial species was examined (Figure 3.14). Stage 11 *Drc-GFP* embryos were injected either with *DH5α E. coli* (OD=1; Figure 3.14A) or *Ecc15* (OD=1; Figure 3.14B), and their induction of the *Drc* promoter construct was compared to that of control Stage 15 *Drc-GFP* embryos at 6 hours post injection. At 6 hours post infection with *DH5α E. coli*, a stark difference in *Drc-GFP* was observed; whilst control embryos injected at Stage 15 of development displayed an extensive, high level of *Drc-GFP* expression concordant with previous results, no response was evident in those embryos injected Stage 11 (Figure 3.14A). Despite this difference in *Drc-GFP* expression, and hence the inferred differential immune competence of early and late stage embryos, both cohorts of embryos continued to proceed through the stereotypical events of embryogenesis, as implied by the invagination of the gut in the embryos injected at Stage 15 and yolk sac morphology those injected at Stage 11 (Figure 3.14A). Results at this time-point exhibited by embryos injected with *Ecc15* show a similar trend; whilst embryos injected at Stage 15 of development show a robust, extensive *Drc-GFP* response, embryos injected at Stage 11 do not exhibit any expression of the transgene although gross development appears unaffected (Figure 3.14B).

Furthermore, this lack of immune capability within the Stage 11 embryo was demonstrated by their decreased viability post infection with a variety of Gram-negative and –positive bacterial species (Figure 3.14C). Groups of ≥100 Stage 15 or Stage 11 embryos were selected and received either control sterile endoxtoxin-free PBS treatment, or injection with *DH5α E. coli* (OD=1), *Mg1655 E. coli* (OD=1), *M. luteus* (OD=1) or *Ecc15* (OD=0.1). Although Stage 11 embryo survival to PBS injection was not significantly different to that of the Stage 15 embryo, infection of Stage 11 embryos with bacterial species significantly decreased their survival to first instar larval stages. Whilst the

reduction in viability to non-pathogenic *DH5α E. coli* infection was relatively minor, although significant, in comparison to Stage 15 embryo survival levels (62%±3.54, p≤0.05), infection with *Mg1655 E. coli*, *M. luteus* and *Ecc15* highly and significantly decreased Stage 11 embryo viability compared to that of Stage 15 controls (20%±4.73, p≤0.01, 39%±13.6, p≤0.001 and 24%±7.00, p≤0.01 respectively). These latter results are concordant with viability levels observed when Stage 15 rel^{e20} and $modSP^{1}$ embryos infected with *Ecc15* and *M. luteus* respectively (Figure 3.10D), further inferring that embryos infected at Stage 11 of development behave similarly to embryos afflicted with a compromised systemic immune response. Thus not only do earlier stage embryos demonstrate a lack of initial immune competence when resolving bacterial infections, but this inability may also lead to their eventual demise, with a dramatic reduction in embryo viability.

Moreover, this reduction in viability is not due to prolonged overstimulation of the Stage 11 immune system, but a specific inability of embryos at this developmental stage to mount an effective response. Injection of heat-killed *Ecc15* (OD=1) into Stage 11 embryos does not appear to affect their subsequent viability (Figure 3.14D); in fact, the percentage of Stage 11 embryos reaching the first larval instar stage upon injection with heat-killed *Ecc15* is not significantly different to that of PBS injected Stage 11 embryos (p≥0.05; Figure 3.14E). Thus heat-killing *Ecc15* prior to infection reverses its detrimental effects on Stage 11 embryo viability, suggesting that the prolonged presence and potential recognition of high levels of pathogen alone does not affect early stage survival. This evidence is further supported by the fact that genetic inactivation of IMD signaling upon *Ecc15* infection (OD=1), by mutation of *PGRP-LC* ($PGRP\text{-}LC^{E12}$), does not improve Stage 11 embryo viability compared to that of WT Stage 11 embryos (Figure 3.14E). If anything, Stage 11 $PGRP\text{-}LC^{E12}$ viability is significantly decreased upon *Ecc15* infection.

In addition, live embryos containing a PGRP-LC-GFP transgene were imaged at different stages using confocal microscopy; to determine if a difference in PGRP-LC expression could be observed throughout embryogenesis to further

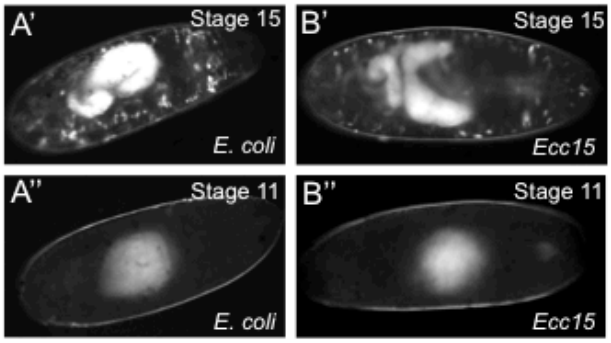
corroborate the notion that early stage embryos were less immune competent than those at late stages. No expression of PGRP-LC-GFP was exhibited in Stage 10 embryos (Figure 3.14F). Conversely, upon reaching Stage 13 of embryogenesis, weak PGRP-LC-GFP expression was detected, localized to epidermal cell membranes (Figure 3.14F). This PGRP-LC-GFP expression was further enhanced in the Stage 15 embryos, showing a more intense signal. This would suggest embryonic immune competence would originate at approximately mid-embryogenesis, potentially involving PGRP-LC. As such, the biological event which regulates this process must occur within this developmental time window.

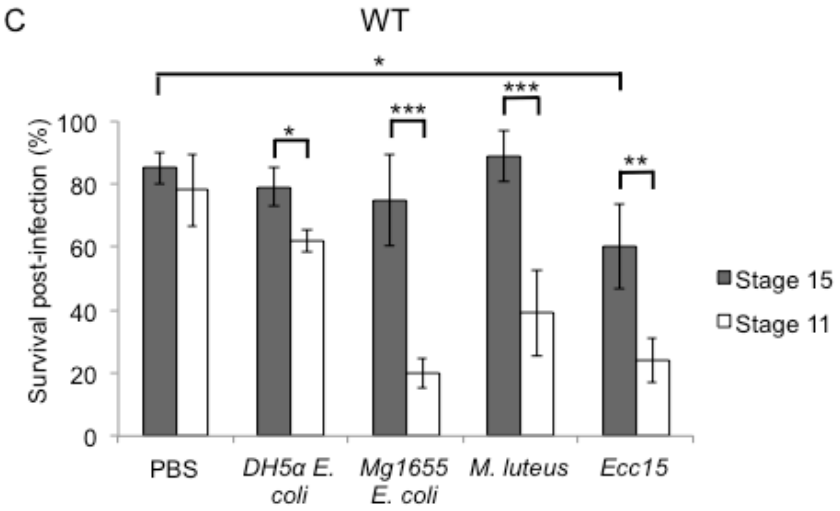
Taken together, these results suggest that Stage 11 embryos are immunologically immature; they do not appear to be able to mount the robust immune response to bacterial challenge demonstrated at later stages of embryogenesis. Moreover, this inability to respond to infection likely results in their increased mortality to infection. This is corroborated by the lack of PGRP-LC expression within these embryos until Stage 13 of development, suggesting that the origin of embryonic immune competence arises during mid-embryogenesis.

Figure 3.14: Stage 11 embryos show a reduced immune competency in response to bacterial invasion

(**A-B**) Stage 15 and Stage 11 *Drc-GFP* embryos were injected with either *E. coli* or *Ecc15*, and the expression of *Drc-GFP* observed at 6 hours post injection (≥25 embryos per experiment). At 6h post injection, no *Drc-GFP* expression was apparent in Stage 11 embryos injected with either *DH5α E. coli* (**A''**) or *Ecc15* (**B''**), in contrast to the robust response of Stage 15 controls (**A' and B'**). (**C**) Bar graph showing mean percentage survival of Stage 15 and Stage 11 WT embryos post infection with a range of bacterial species, as well as after microinjection with PBS. Stage 11 embryos infected with all bacterial species examined showed significantly reduced survival in comparison to the survival of Stage 15 controls (Unpaired T-Test, n=3, with ≥ 100 embryos injected per experiment). (**D**) Bar graph showing mean percentage survival of Stage 11 embryos post injection with sterile endotoxin free PBS, live *Ecc15* (OD=0.1) or heat-inactivated *Ecc15* (OD=0.1; *Ecc15* HI). The effects of *Ecc15* on Stage 11 embryo mortality can be reversed by heat-inactivation of bacteria, suggesting that Stage 11 embryo viability is reduced by an inability to eliminate infection and not chronic overstimulation of the immune system. (**E**) Bar graph showing mean percentage survival of Stage 15 and Stage 11 *PGRP-LC*^{E12} mutant embryos upon PBS or *Ecc15* infection (OD=0.1). *Ecc15* infection significantly reduced Stage 11 survival to infection in comparison to PBS injected ($p \leq 0.001$) and to Stage 15 *Ecc15* injected ($p \leq 0.01$) levels, indicating that overstimulation of the Stage 11 immune system is not responsible for the increased embryo mortality observed. (**F**) Live imaging of *PGRP-LC-GFP* embryos. PGRP-LC-GFP fusion protein was not detected until Stage 13 of embryogenesis, albeit at very low levels. This expression was enhanced at Stage 15 of development, with PGRP-LC-GFP clearing exhibiting localization at the cell membrane of epidermal cells (n=3). Scale bars represent 20μm.

* Indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$. All other changes do not show statistical significance. Error bars represent standard deviation.


A'
Stage 15
E. coli
B'
Stage 15
Ecc15
A''
Stage 11
E. coli
B''
Stage 11
Ecc15


C
WT
Survival post-infection (%)
100
80
60
40
20
0
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***
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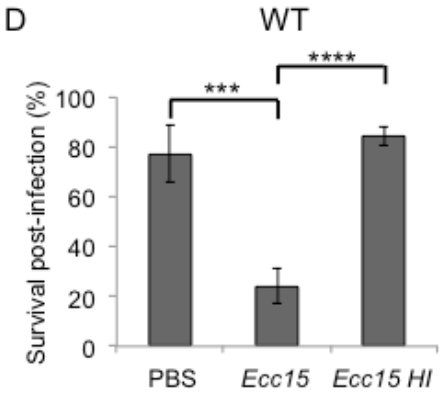
PBS
DH5α E. coli
Mg1655 E. coli
M. luteus
Ecc15
Stage 15
Stage 11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WT
Survival post-infection (%)
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80
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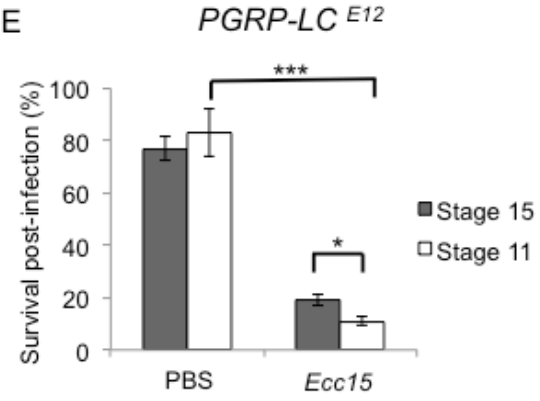
Ecc15 HI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Survival post-infection (%)
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PBS
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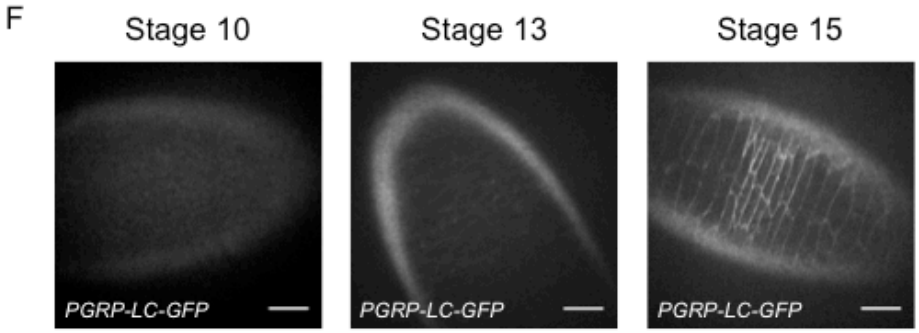
Stage 15
Stage 11


F
Stage 10
Stage 13
Stage 15
PGRP-LC-GFP
PGRP-LC-GFP
PGRP-LC-GFP

3.2.8. 20-Hydroxyecdysone signaling is required for Stage 15 embryo AMP gene induction and survival to infection

Having observed that embryos infected at Stage 11 lacked the immune competence demonstrated at later stages of development, we postulated that this differential ability may arise as a result of the 20-hydroxyecdysone (20-HE) pulse, which occurs at around Stage 12 of development (Kozlova and Thummel, 2003). Since 20-HE signaling is crucial for embryonic development, we first verified if Stage 15 embryos containing mutations in the ecdysone receptor (EcR) were viable, and moreover if they were more susceptible to infection than WT controls. To assess embryo viability, non-infected Stage 15 heterozygous (*EcR*^{Q50st}*/CTG*, *EcR*^{M554fs}*/CTG*), homozygous (*EcR*^{Q50st}*/EcR*^{Q50st}, *EcR*^{M55fs}*/EcR*^{M554fs}) and transheterozygous (*EcR*^{Q50st}*/EcR*^{M554fs}) *EcR* mutant embryos were selected and monitored for development to first instar larvae (Figure 3.15A). *EcR*^{Q50st} is a mutation selective to the EcR-B1 isoform, whereas that of the *EcR*^{M554fs} mutant is in a common exon, and thus affects all three EcR isoforms (Bender *et al.,* 1997). The viability of Stage 15 embryos whereby a dominant-negative form of EcR-B1 was expressed using the Gal4-UAS system either ubiquitously (*DaGal4, UAS EcR-B1 DN*) or selectively in either hemocytes (*srpGal4, UAS EcR-B1 DN*), epithelia (*E22cGal4, UAS EcR-B1 DN*) or tracheal cells (*BtlGal4, UAS-EcR-B1 DN*) was also assessed (Figure 3.15A). Both heterozygous and transheterozygous mutant embryos, as well as those expressing the dominant negative form of EcR-B1 in hemocytes or tracheal cells, did not show a significant difference in viability in comparison to WT embryos. In contrast, *EcR*^{M55fs} homozygous mutants exhibited a dramatic decrease in viability compared to WT controls, with 12%±2.65 of embryos selected hatching into first instar larvae ($p \leq 0.0001$; Figure 3.15A). A similar effect was noted with *DaGal4, UAS EcR-B1 DN* and *E22CGal4, UAS EcR-B1 DN* embryos (21%±17.5, $p \leq 0.001$ and 25%±13.6 respectively). These results are not surprising given the ubiquitous expression of EcR isoforms within the embryo by Stage 12 of development, which is particularly enriched in the amnioserosa (Figure 3.16A-B). However, whilst *EcR*^{Q50st} homozygotes did display a significantly different viability to WT controls ($p \leq 0.05$), a high

proportion of these embryos survived to first instar larval stages (86% ± 4.36) and hence could be utilized in further studies.

Therefore, the susceptibility of Stage 15 *EcR*^{Q50st} homozygous, *EcR*^{Q50st}/*EcR*^{M554fs} transheterozygous, *srp>EcR-B1 DN* and Btl>EcR-B1 DN embryos to *Ecc15* infection (OD=0.1) was tested (Figure 3.15B). Survival of *EcR*^{Q50st} homozygous and *EcR*^{Q50st}/*EcR*^{M554fs} transheterozygous mutant embryos was significantly compromised by *Ecc15* infection compared to WT survival at 24 hours post injection ($p \leq 0.01$ and $p \leq 0.05$ respectively), similar to the viability of embryos injected with *Ecc15* at Stage 11 of development (Figure 3.15B). Moreover, *EcR*^{Q50st} homozygous embryos also displayed a small yet significant decrease in viability upon sterile PBS injection alone ($p \leq 0.05$), although this was not the case for the *EcR*^{Q50st}/*EcR*^{M554fs} transheterozygous mutant embryos. In terms of the location that 20-HE signaling is required to mediate effective embryonic survival to infection, no significant difference in viability was observed between WT and *srp>EcR-B1 DN* embryos (Figure 3.15B), inferring that hemocytes are not required for perpetuating 20-HE signaling in this process. Interestingly, the viability of *Btl>EcR-B1 DN* embryos was demonstrated to be selectively reduced upon Ecc15 infection in comparison to WT embryos (Figure 3.15B), inferring that the presence and functionality of the EcR-B1 isoform in tracheal tissue is required to mediate embryonic survival to *Ecc15* infection.

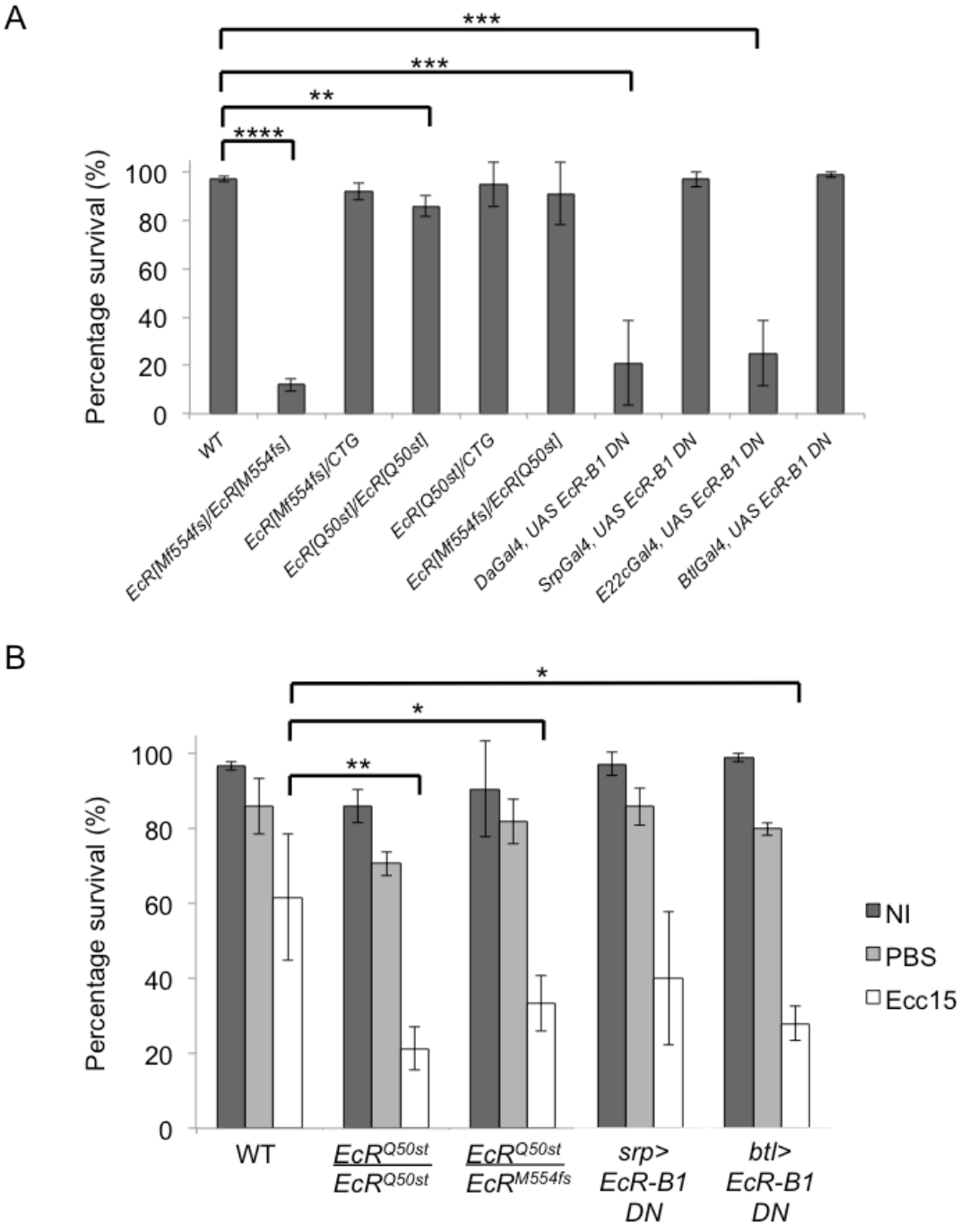
Subsequently, we can postulate that 20-HE signaling is integral in the embryonic systemic immune response to bacterial infection, and may also play a role in resolution of mechanical damage. Moreover, embryos unable to mediate effective 20-HE signaling phenocopy the lack of immune capability observed in infected Stage 11 embryos, suggesting that 20-HE may play a role in the maturation of the systemic immune system in *Drosophila* embryos. In particular, 20-HE signaling in tracheal cells appears to play a crucial role in this process.

To determine if 20-HE signaling was required for AMP gene induction upon infection, infection studies were performed with Stage 15 *EcR*^{Q50st} homozygous mutant embryos and their resulting AMP gene expression assessed via RT-qPCR. Compared to WT control embryos, specific AMP gene activity was severely and significantly downregulated upon infection with *Ecc15* (OD=1) in *EcR*^{Q50st} mutants (Figure 3.17). *CecA1, Def,* and *Mtk* all demonstrated a lack of induction upon *Ecc15* infection within the *EcR*^{Q50st} mutant background (Figure 3.17A). As such, relative levels of expression for this subset of AMPs upon *Ecc15* infection was not significantly different to those stimulated by PBS treatment alone (Figure 3.17A), thus suggesting that the direct effects of *Ecc15* upon AMP stimulation were effectively disrupted in *EcR*^{Q50st} mutants. Interestingly, the expression of other AMP genes could still be induced in *EcR*^{Q50st} mutants, experiencing a significant upregulation upon *Ecc15* treatment, including *Drc* and *AttA* (Figure 3.17B). Moreover, no significant difference in *Dpt* expression was noted between WT control and *EcR*^{Q50st} mutant embryos (Figure 3.17B). However, when comparing the basal levels of *Dpt, Drc* and *AttA* expression in non-infected WT and *EcR*^{Q50st} embryos, the levels of gene expression are significantly decreased in *EcR*^{Q50st} mutants (Figure 3.17C), suggesting that *EcR*-deficient embryos are unable to maintain basal levels of these AMPs and are hence compromised even before an infection scenario. Therefore, in Stage 15 embryos 20HE signaling is required for the expression of a subset of AMP genes, and potentially to maintain the basal levels of AMP gene expression.

Figure 3.15: 20-Hydroxyecdysone is crucial for the regulation of the *Drosophila* immune response.

(**A**) Quantification of viability of non-infected Stage 15 *Ecdysone receptor* (*EcR*) mutant embryos and Stage 15 embryos expressing a dominant-negative form of EcR-B1. Embryos that were heterozygous or transheterozygous for either *EcR* mutation, as well as embryos where a dominant-negative form of the EcR-B1 was expressed selectively in hemocytes or tracheal cells under the control of the *serpent* (*srp*) or breathless (*btl*) promoters respectively, did not show significantly different viability to WT embryos. Homozygous mutant embryos and those where dominant-negative EcR-B1 was ubiquitously expressed using the *daughterless* (*da*) or *E22c* promoters showed highly significantly reduced viability (P≤0.05, Unpaired T-Test, n=3 where each replicate consisted of ≥50 embryos). (**B**) Bar graph showing the effect of bacterial infection upon the survival of Stage 15 *EcR* mutant embryos. The viability of EcR^{Q50st} homozygous and EcR^{Q50st} /EcR^{M554fs} transheterozygous mutant embryos was significantly reduced upon *Ecc15* infection (OD=0.1) compared to WT controls. The viability of embryos expressing EcR-B1 DN in tracheal cells was also significantly decreased upon *Ecc15* infection compared to WT controls. The survival of embryos expressing dominant-negative EcR-B1 receptor in hemocytes (*srp>EcR-B1 DN*) was not significantly different from that of WT embryos, indicating that hemocytes may not play a role in perpetuating 20-HE signalling for the maturation of the embryonic immune system (Unpaired T-Test, N=3 where each replicate comprises ≥100 embryos).

* indicates p ≤ 0.05, ** indicates p ≤ 0.01; *** indicates p ≤ 0.001; **** indicates p ≤ 0.0001


A
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Percentage survival (%)
100
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60
40
20
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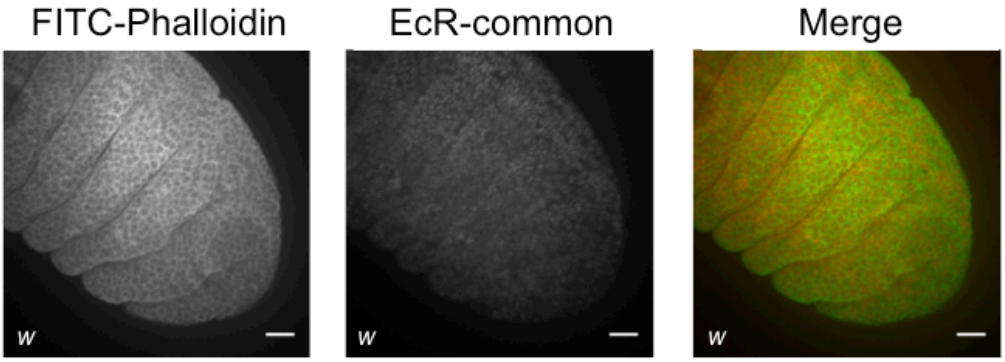
WT
EcR[Mf554fs]/EcR[M554fs]
EcR[Mf554fs]/CTG
EcR[Q50st]/EcR[Q50st]
EcR[Q50st]/CTG
EcR[Mf554fs]/EcR[Q50st]
DaGal4, UAS EcR-B1 DN
SrpGal4, UAS EcR-B1 DN
E22cGal4, UAS EcR-B1 DN
BtlGal4, UAS EcR-B1 DN
B
*
*
**
Percentage survival (%)
100
80
60
40
20
0
WT
EcRQ50st / EcRQ50st
EcRQ50st / EcRM554fs
srp> EcR-B1 DN
btl> EcR-B1 DN
NI
PBS
Ecc15

Figure 3.16: The Ecdysone Receptor is expressed ubiquitously in *Drosophila* embryos

(**A**) Ventral images of the anterior region of Stage 12 embryos co-immunostained for all isoforms of the ecdysone receptor (EcR; red) and for FITC-phalloidin (green). All cells within the embryonic epidermis appeared to contain EcR, localized to the cell cytoplasm (n=3). (**B**) Lateral images of Stage 12 embryos stained co-immunostained as above. The localisation of EcR protein was noted to be particularly enhanced within the amnioserosa of stained Stage 12 embryos (n=3). Scale bars represent 15μm.

A


FITC-Phalloidin
EcR-common
Merge
w
w
w

B

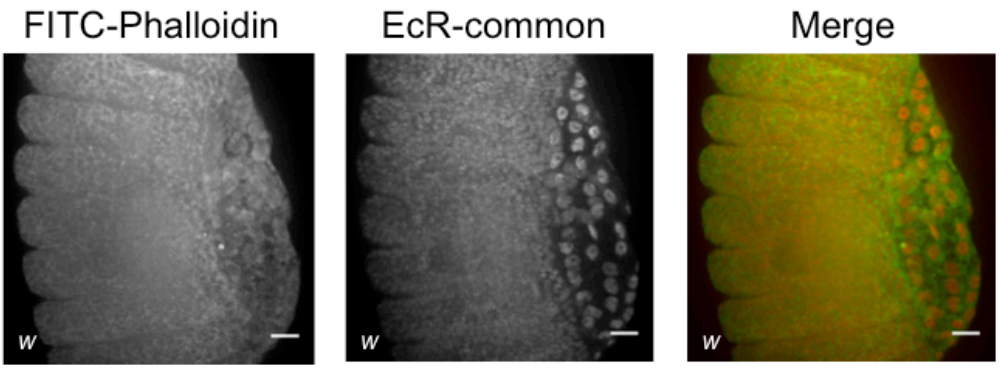
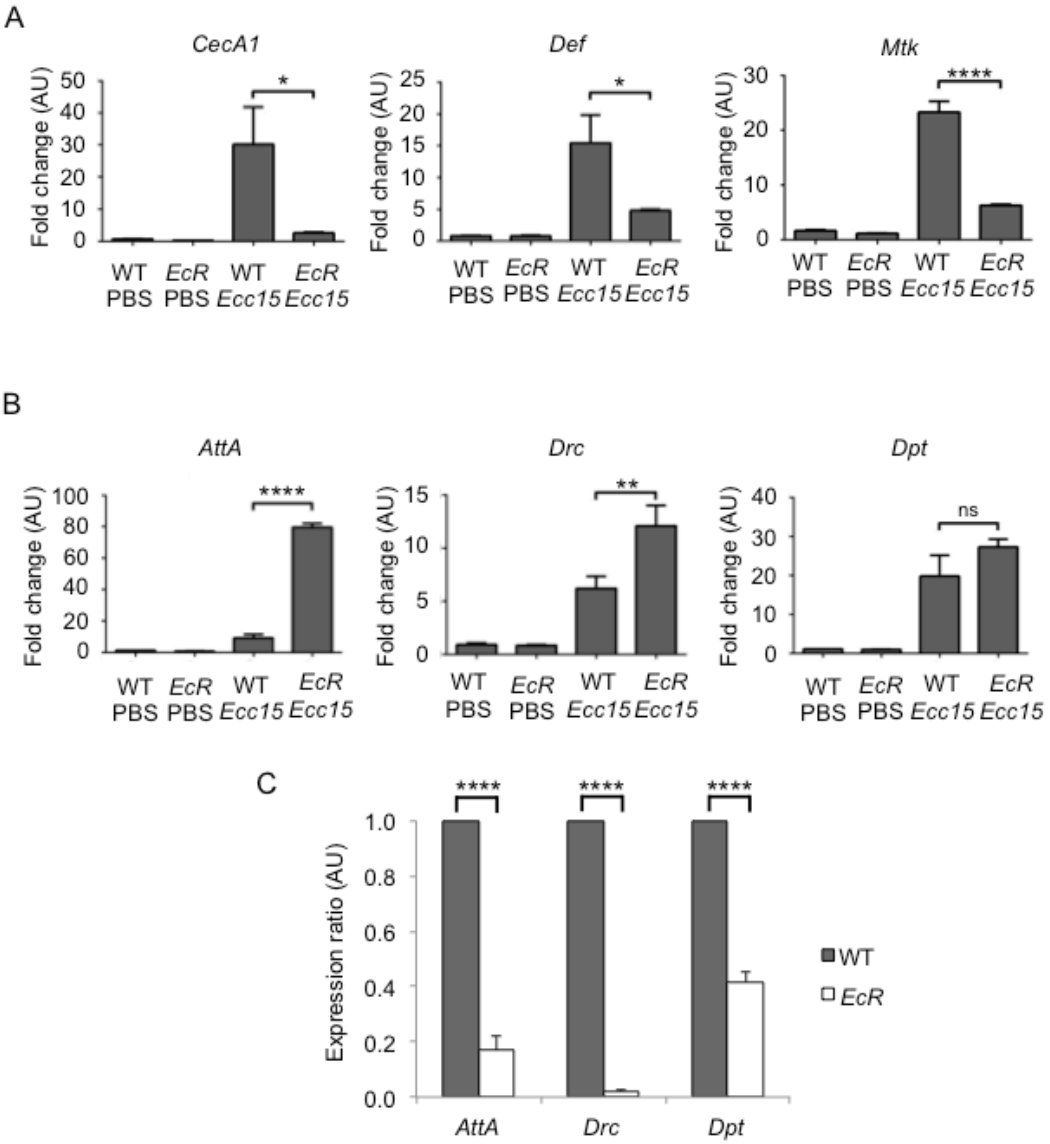

FITC-Phalloidin
EcR-common
Merge
w
w
w

Figure 3.17: AMP gene induction is partially disrupted in *EcR* homozygous mutant embryos

(**A-B**) Bar graphs showing quantification of AMP gene expression levels in EcR^{Q50st} homozygous mutant embryos versus WT controls upon sterile endotoxin-free PBS and *Ecc15* injection by RT-qPCR. Relative expression levels in *Ecc15* treated embryos were normalised to PBS-injected levels for both WT and mutants. *CecA1, Def, Drs* and *Mtk* expression was significantly inhibited in EcR^{Q50st} mutants compared to WT controls (**A**; One-way ANOVA, n=3 where ≥200 embryos were used per experiment). Conversely, expression of *AttA* and *Drc* was significantly increased in EcR^{Q50st} mutants compared to WT embryos upon *Ecc15* infection (**B**). No significant difference in *Dpt* expression was noted when comparing WT and EcR^{Q50st} mutant levels (**B**). * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$; **** indicates $p \leq 0.0001$. All other changes do not show statistical significance.


A
CecA1
Def
Mtk
Fold change (AU)
WT PBS
EcR PBS
WT Ecc15
EcR Ecc15
*
*
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B
AttA
Drc
Dpt
****
**
ns
C
Expression ratio (AU)
AttA
Drc
Dpt
WT
EcR

3.2.9. 20-HE treatment rescues Stage 11 embryo AMP expression

To further investigate the role of 20-HE in the maturation of embryonic systemic immunity, Stage 11 *Drc-GFP* embryos were co-injected with *Ecc15* (OD=0.5) and 20-HE, to test whether treatment with 20-HE could rescue *Drc-GFP* expression and survival at this stage of embryogenesis (Figure 3.18A-B). The percentage of embryos expressing *Drc-GFP* upon *Ecc15* and 20-HE co-administration (*Ecc15*+20-HE) was compared to that of those Stage 11 embryos receiving *Ecc15*, 20-HE or sterile PBS treatment alone. These results were subsequently compared to those of Stage 15 embryos receiving identical treatments. Intriguingly, upon *Ecc15*+20-HE treatment, a significantly increased percentage of Stage 11 *Drc-GFP* embryos were able to respond to *Ecc15* infection upon co-administration of 25μM 20-HE when compared to that of Stage 11 embryos treated with *Ecc15* alone (30%±4.0 compared to 12%±4.9 respectively, Figure 3.17A and B). Conversely, it should be noted that this rescue is not complete; the level of *Drc-GFP* response in rescued embryos does not parallel that of Stage 15 counterparts in terms of the percentage of embryos able to respond to infection (Figure 3.18B). Nevertheless, those Stage 11 embryos that are effectively rescued exhibit a response that appears qualitatively similar to Stage 15 counterparts (Figure 3.18A), with a similar degree and localization of *Drc-GFP* expression.

However, 20-HE itself does not appear to significantly induce *Drc-GFP* expression as both Stage 15 and Stage 11 embryos receiving 20-HE treatment alone did not significantly respond compared to sterile PBS control groups (Figure 3.18A and B). Similarly, co-administration of *Ecc15*+20-HE to immune competent Stage 15 embryos did not significantly increase the percentage of embryos able to respond to bacterial stimuli at this stage of development, suggesting that there is no further immune advantage to applying 20-HE at Stage 15 of development (Figure 3.18B). Nevertheless, it is worth noting that both PBS and 20-HE treatment themselves did stimulate a low but significant level of *Drc-GFP* response in Stage 15 embryos (12%±1.4 and 8%±0.71 respectively; Figure 3.18B), which could be attributed to the damage of injection process. Interestingly, this effect is abolished in Stage 11

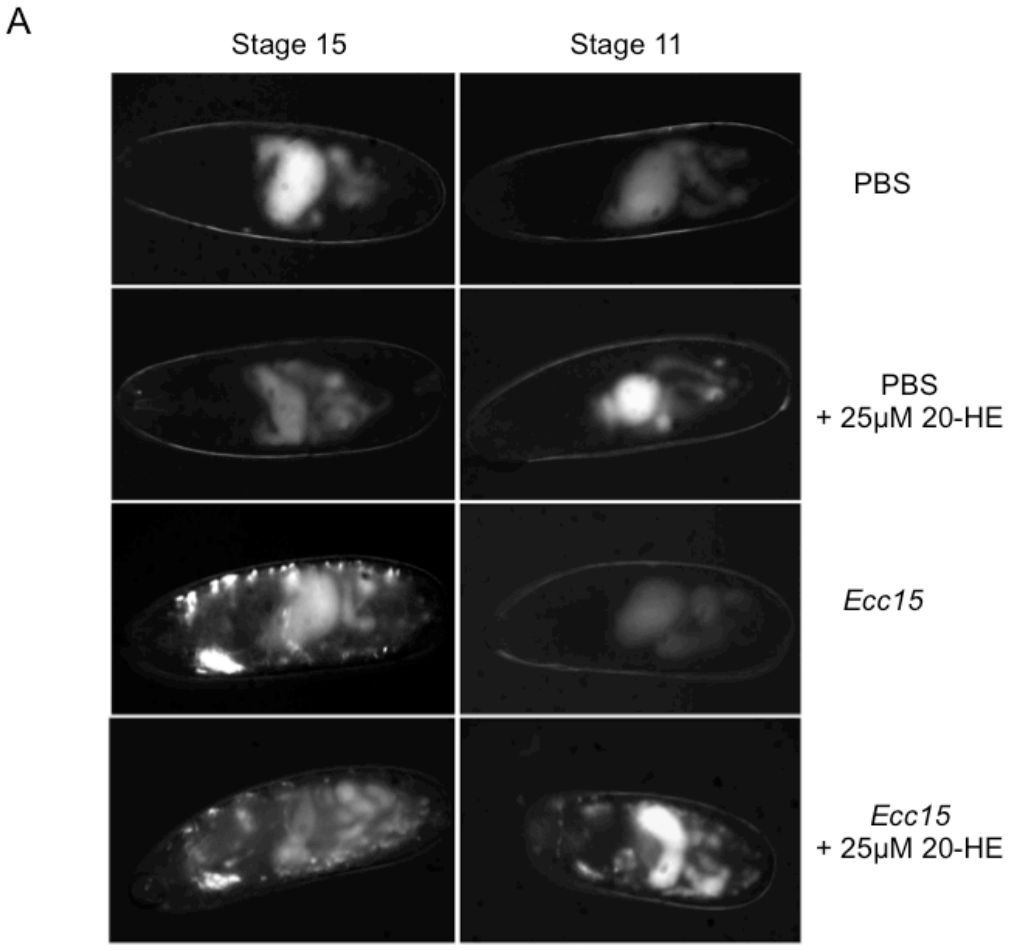
embryos, permitting the inference that the AMP response to damage at this stage is also inhibited and thus 20-HE is also able to mediate AMP expression in response to damage alone.

In terms of a developmental advantage, given the role of 20-HE in mediating crucial developmental processes, those embryos receiving 20-HE treatment did not appear to display perturbations in developmental timing (Figure 3.19). At 12 hours post injection, no significant difference in the percentage of Stage 11 embryos reaching advanced stages of embryogenesis was noted between any treatment groups, as determined by gut morphology ($p \geq 0.05$, Figure 3.19A). However, treatment with 20-HE upon *Ecc15* infection did not rescue Stage 11 embryo survival to first larval instar stages. (Figure 3.19B), with only 9.3%±2.3 versus 12%±.4.0 survival for *Ecc15*+20-HE and *Ecc15 t*reated groups respectively. The viability of these groups was not significantly different ($p \geq 0.05$, Unpaired T-Test), despite 20-HE treatment, and both groups still remained highly significantly different to PBS and PBS+20-HE treated controls ($p \leq 0.0001$, Unpaired T-Test). Therefore, whilst 20-HE treatment may rescue the *Drc-GFP* expression of Stage 11 embryos, it does not confer any benefit to Stage 11 embryo viability upon *Ecc15* infection.

Taken together, these results indicate that the immune immaturity of Stage 11 embryos can be rescued by application of 20-HE, thus demonstrating a direct role for this hormone in the maturation of the embryonic immune response. However, 20-HE is not able to induce immune activation itself, requiring a bacterial co-stimulus, although there may be a potential role for 20-HE in mediating a minor response to damage signals. In any case, there does not appear to be any developmental benefit or cost of administration of exogenous 20-HE treatment itself, and furthermore 20-HE does not protect against *Ecc15*-mediated killing of Stage 11 embryos despite a partial rescue of the AMP response.

Figure 3.18: 20-HE treatment rescues the AMP response in Stage 11 embryos

(**A-B**) Quantification and images of the rescue of Stage 11 embryos with 20-hydroxyecdysone (20-HE). (**A**) Ventral images of corresponding Stage 11 and Stage 15 *Drc-GFP* embryos injected with *Ecc15* +/- 20-HE. A rescue of Drc-GFP expression was noted in Stage 11 embryos receiving 20-HE treatment upon Ecc15 infection. (**B**) Graphs showing expression of Drc-GFP transgene in Stage 11 and Stage 15 *Drc-GFP* embryos at 12h post infection with *Ecc15* +/- co-injection with 20-HE. A significantly larger proportion of Stage 11 embryos that received *Ecc15*+20-HE treatment expressed *Drc-GFP* at 12h post injection than those receiving *Ecc15* treatment alone ($p \leq 0.001$, Unpaired T-Test, N=3 with ≥100 embryos injected per experiment). In terms of the proportion of responsive embryos, 20-HE treatment did not rescue Drc-GFP expression to Stage 15 levels. Moreover, the proportion of Stage 11 embryos responding to PBS or PBS+20-HE treatment alone was significantly less than their Stage 15 counterparts. ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$.


A
Stage 15
Stage 11
PBS
PBS
+ 25μM 20-HE
Ecc15
Ecc15
+ 25μM 20-HE

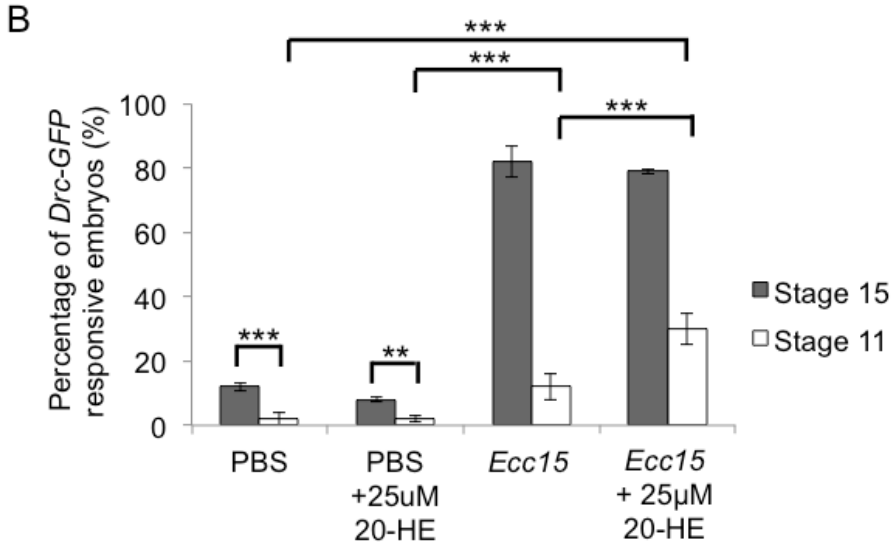
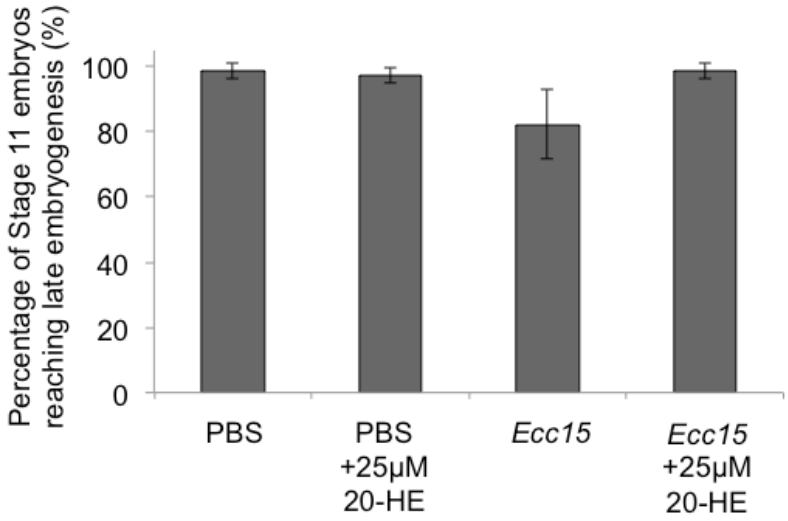

B
Percentage of Drc-GFP responsive embryos (%)
100
80
60
40
20
0
***
***
***
***
**
Stage 15
Stage 11
PBS
PBS +25uM 20-HE
Ecc15
Ecc15 + 25μM 20-HE

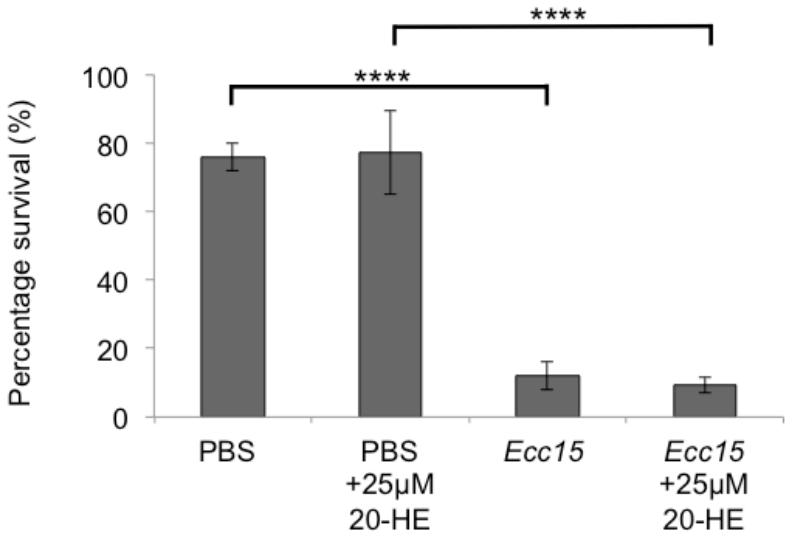
Figure 3.19: 20-HE does not confer any developmental or survival benefit on Stage 11 embryos infected with *Ecc15*

(**A-B**) Cohorts of Stage 11 embryos were treated with either PBS or *Ecc15* (OD=0.5) +/- 20-HE and their development assessed by gut morphology at 12 hours post treatment. Their subsequent survival to first larval instar stage was also observed at 30 hours post treatment (n=3, where ≥50 embryos were employed per replicate). No significant difference was observed in the percentage of Stage 11 embryos reaching advanced stages of embryogenesis (Stage 16 onwards) between any of the treatment groups (**A;** $p \geq 0.05$), suggesting that 20-HE does not confer any developmental cost or benefit when administered to Stage 11 embryos in its own right or upon *Ecc15* treatment. Injection of 20-HE also does not promote the survival of Stage 11 embryos infected with *Ecc15* (**B**). No significant change in viability was noted between *Ecc15* and *Ecc15*+20-HE treated groups ($p \geq 0.05$, Unpaired T-Test, n=3) and the viability of these groups remained significantly reduced from PBS and PBS+20-HE controls respectively ($p \leq 0.0001$; Unpaired T-Test, n=3), **** indicates $p \leq 0.0001$. All other comparisons should be considered as not significant.

A


Percentage of Stage 11 embryos reaching late embryogenesis (%)
100
80
60
40
20
0
PBS
PBS +25µM 20-HE
Ecc15
Ecc15 +25µM 20-HE

B


****
****
Percentage survival (%)
100
80
60
40
20
0
PBS
PBS +25µM 20-HE
Ecc15
Ecc15 +25µM 20-HE

3.2.10 20-HE signalling is required to control bacterial proliferation upon infection

Since administration of 20-HE rescued AMP expression but not the viability of Stage 11 embryos infected with *Ecc15*, it was necessary to assess if bacteria were effectively prosecuted under these conditions. To determine if *Ecc15* were effectively eliminated within Stage 11 embryos, cohorts of Stage 11 and control Stage 15 embryos were injected with *Ecc15* (OD=0.1), incubated for 7.5 hours to allow for bacterial proliferation and subsequently crushed and the resulting suspension spread on LB plates to assess the numbers of colony forming units (CFUs) that could be isolated from each test group. Ratios of CFU counts between control and test groups were then calculated to determine if changes in bacterial burden were significant (Figure 3.20).

Results indicated that 7.5 hours after the initial infection, Stage 11 embryos harboured more bacteria than Stage 15 control embryos (Figure 3.20A); this would thus indicate that Stage 11 embryos are not able to eliminate bacteria as effectively as their more robustly immune Stage 15 counterparts. However, due to variability in CFU counts from infected Stage 11 embryos, this difference is not significant ($p=0.08$, Unpaired T-Test). Moreover, it is important to note that the bacterial burden in Stage 15 embryos was not reduced to undetectable levels (Figure 3.20A), suggesting that even immune competent embryos were not able to effectively clear the *Ecc15* infection 7.5 hours after the initial administration. Nonetheless, treatment of Stage 11 embryos with 20-HE upon *Ecc15* infection significantly reduces the bacterial burden contained within these embryos compared to controls receiving only an *Ecc15* infection ($p\leq0.001$; Figure 3.20B). Interestingly, this effect can also be replicated in Stage 15 embryos, which also experienced a highly significant decrease in bacterial burden after an initial co-administration with 20-HE upon *Ecc15* infection ($p\leq0.0001$, Figure 3.20B), implying that 20-HE is can mediate effective inhibition of bacterial proliferation within the *Drosophila* embryo.

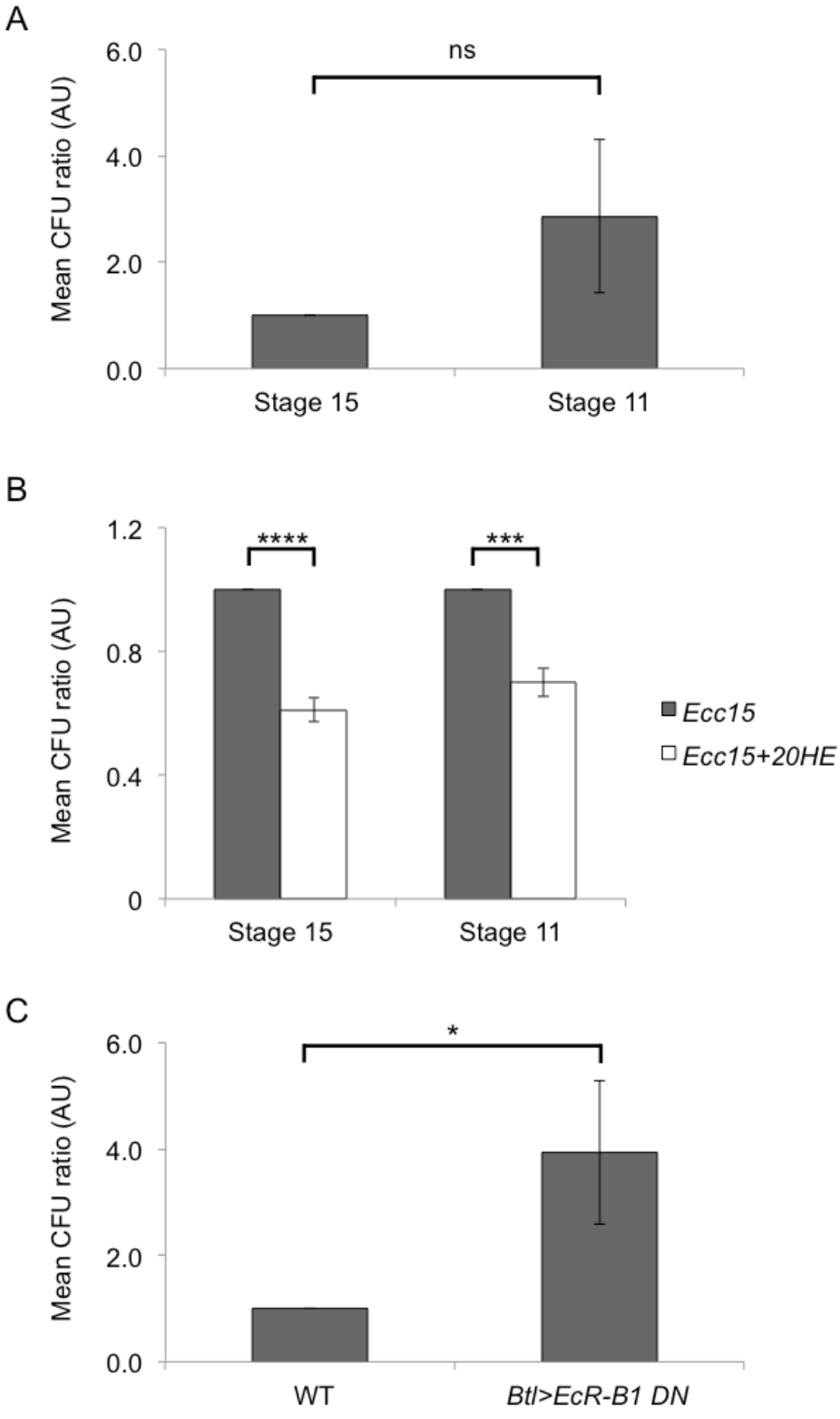
Most interestingly, upon assessing the *Ecc15* burden in Stage 15 embryos expressing a dominant negative form of the EcR-B1 isoform in trachea cells it

was discovered that these embryos harboured a significantly greater number of bacteria at 7.5 hours post infection than their WT counterparts ($p \leq 0.05$, Unpaired T-Test; Figure 3.20C), inferring the importance of 20-HE signaling in the Stage 15 immune response. This effect on *Ecc15* burden phenocopied that observed in Stage 11 embryos (Figure 3.20A), providing a link between 20-HE signaling and the immune incompetence exhibited by Stage 11 embryos. Moreover, it again reinforced the importance of the trachea in mediating an effective embryonic immune response to bacterial infection.

Therefore, these results emphasize the importance of 20-HE signaling in effective prevention of bacterial proliferation *in vivo*. More specifically, they suggest that 20-HE signaling in the trachea is critical to facilitate the maturation of embryonic immune response and ultimately to prevent the expansion of infection, albeit this does not eradicate the infection in its entirety.

Figure 3.20: 20-HE treatment decreases bacterial load in *Drosophila* embryos

(**A-B**) Groups of Stage 15 and Stage 11 embryos were infected with *Ecc15* (OD=0.1) +/- 25μM 20-HE (n=3, with ≥50 embryos per treatment group per experiment). At 7.5 hours post infection, the embryos were mechanically disrupted and the resulting suspension grown on LB agar plates to recover bacteria and assess the infection burden via counting of colony forming units (CFUs). Stage 11 embryos were determined to have a greater bacterial burden than Stage 15 embryos at this time-point (**A**), but this difference was not significant ($p \geq 0.05$, Unpaired T-Test). Co-administration of 20-HE upon *Ecc15* infection significantly reduced the bacterial burden in both Stage 15 and Stage 11 embryos (**B**), compared to that observed 7.5 hours after *Ecc15* infection alone ($p \leq 0.001$ and $p \leq 0.0001$ respectively, Unpaired T-Test). A significantly greater bacterial burden was also recorded in Stage 15 embryos expressing a dominant negative form of EcR-B1 in the trachea (*btl>EcR-B1 DN*), compared to WT controls (**C**; $p \leq 0.05$), demonstrating that the inhibition of 20-HE signalling in trachea leads to a less effective bacterial clearance. * indicates $p \leq 0.05$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$.


A
6.0
4.0
2.0
0.0
Mean CFU ratio (AU)
ns
Stage 15
Stage 11
B
1.2
0.8
0.4
0
Mean CFU ratio (AU)
****
***
Stage 15
Stage 11
Ecc15
Ecc15+20HE
C
6.0
4.0
2.0
0.0
Mean CFU ratio (AU)
*
WT
Btl>EcR-B1 DN

3.2.11 Damage and *Ecc15* infection downregulate 20-HE synthesis, metabolism and effector gene expression in Stage 15 embryos

Having established that 20-HE is critical for mediating a successful immune response to *Ecc15*, the relative change in expression levels of genes involved in 20-HE biosynthesis and metabolism, as well as those encoding 20-HE signaling effectors, was assessed upon both sterile PBS injection and *Ecc15* infection in Stage 15 WT embryos using RT-qPCR (Figure 3.21). To assess modulation in expression of the candidate genes upon damage alone, relative expression levels in PBS injected embryos were compared to those in non-infected (NI) embryos (Figure 3.21A). Alternatively, to assess the effects of *Ecc15* infection, comparisons in relative expression levels of candidate genes in PBS and *Ecc15* injected embryos were performed (Figure 3.21B).

Comparison between relative expression levels in NI and PBS injected Stage 15 WT embryos revealed that specific genes involved in 20-HE biosynthesis are downregulated upon such damage (Figure 3.21A). For example, expression of the *ptth, phm, nvd* and *ecd*, genes was significantly downregulated in PBS injected embryos compared to NI controls ($p \leq 0.05$). This was not true for all genes involved in 20-HE synthesis; those such as *dare* and *mld* did not exhibit any modulation upon PBS injection. *Eo*, a component of 20-HE metabolism, was also significantly downregulated in PBS injected embryos compared to NI controls. Of the *eip* genes tested, only *eip78C* displayed any modulation upon PBS injection, as *eip71CD* did not exhibit any significant change in relative expression upon treatment. Furthermore, on examination on relative expression levels of the aforementioned candidates in *Ecc15* infected embryos, it was discovered that seven genes showed significant modulation in expression levels compared to PBS injected embryos ($p \leq 0.05$; Figure 3.21B). *Phm, nvd amd eo* expression was shown to be further downregulated upon *Ecc15* infection, when compared to PBS injected expression levels. Moreover, the expression level of *ptth* was significantly increased upon *Ecc15* infection compared to that observed upon PBS injection alone (Figure 3.21B). Other candidate genes, such as *ecd* and *eip78C*, did not show any difference in expression upon *Ecc15* infection

compared to PBS injected levels, suggesting that these genes do not play a role in the immune response to bacterial infection. However, the most interesting feature of the data was that the remaining three genes exhibiting modulation, *dare, mld* and *eip71CD*, were downregulated solely upon *Ecc15* treatment, indicating an infection specific interaction (Figure 3.21B).

To determine if this aforementioned modulation in candidate gene expression was specific to *Ecc15* infection, relative expression levels were subsequently assessed in Stage 15 WT embryos treated with *Mg1655 E. coli*, and similar comparisons performed (Figures 3.21C). Interestingly, only four genes demonstrated a modulation in expression upon *Mg1655 E. coli* infection. Similar to *Ecc15* infection, injection with *Mg1655 E. coli* also resulted in the decreased expression of *dare* and *eo* as well as the increased expression of *ptth* in the Stage 15 embryo, suggesting that these changes in host gene expression are not specific to *Ecc15* infection. However, the magnitude by which *Ecc15* induced these changes was significantly greater, as can be seen in Figure 3.21D. For example, whilst both *Mg1655 E. coli* and *Ecc15* upregulated *ptth* expression, the change in expression was significantly greater in *Ecc15* infected embryos (Figure 3.21D).

Aside from these differential levels of gene modulation, more dramatic differences can also be observed. For instance, in contrast to the reduced expression noted with *Ecc15* infection, *Mg1655 E. coli* induced increased expression of *mld* (Figure 3.21C). Furthermore, *phm, nvd* and *eip71CD* expression levels upon *Mg1655 E. coli* infection were not significantly different to those observed in PBS injected embryos, in contrast to the host response observed to *Ecc15* infection. This would suggest that different Gram-negative infections can differentially impact 20-HE signaling, and that *Ecc15* infection in particular has a more significant impact on the 20-HE signaling in its embryonic host than *Mg1655 E. coli.*

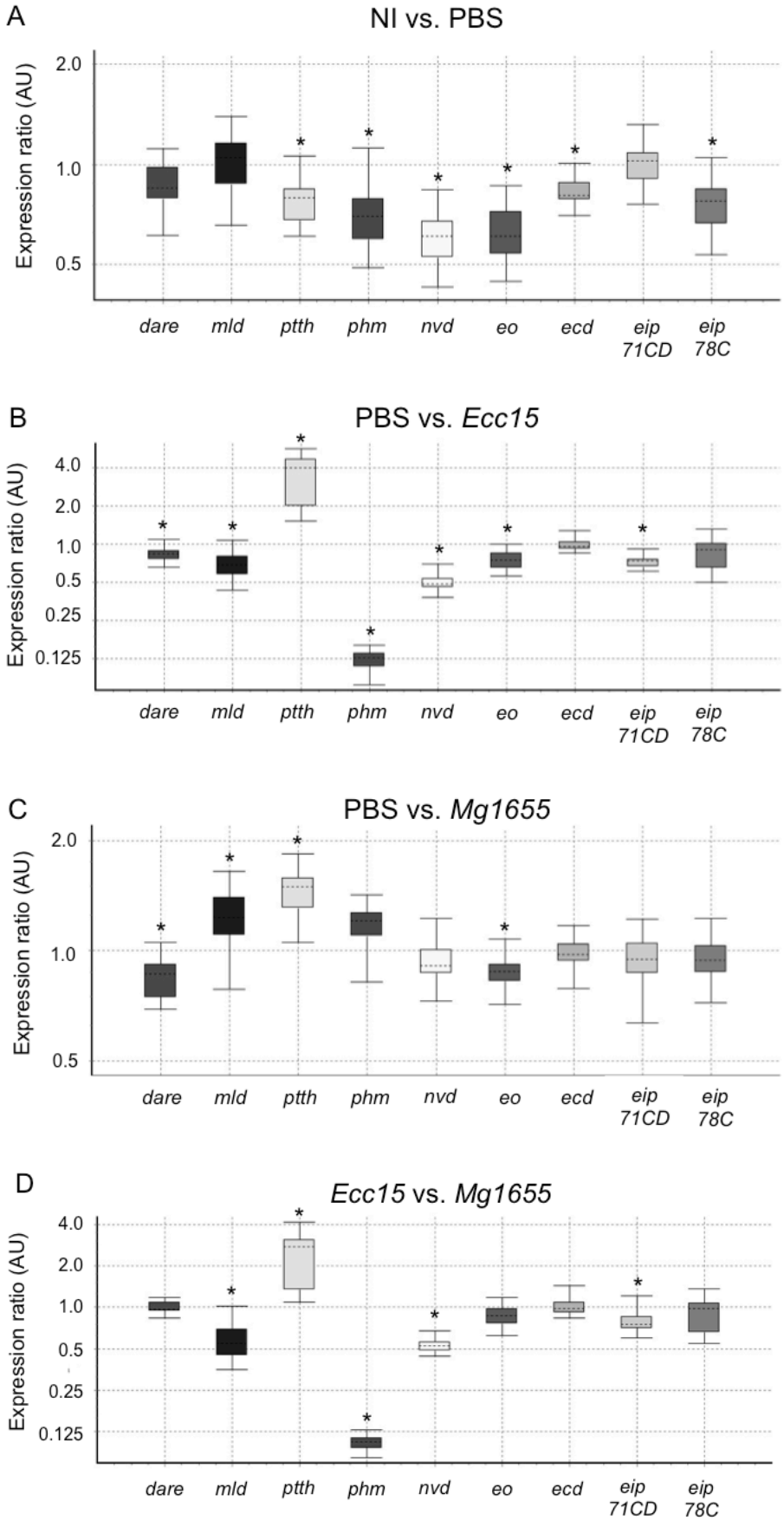
However, not all candidate genes displayed such significant changes in expression upon treatment and as such were not as amenable to analysis. For instance, the average efficiency values of *usp* and *Eip74EF* primer pairs were

lower than acceptable thresholds set for RT-qPCR experiments (see Appendix 6), and these candidate genes were also subsequently excluded from analysis.

To summarise, both damage and infection with Gram-negative bacterial species are able to modulate expression of genes involved in 20-HE signaling within the Stage 15 embryo, with each producing a unique response. Damage induced by PBS injection downregulated a number of genes encoding a diverse variety of 20-HE signaling components. Infection with either *Ecc15* or *Mg1655 E. coli* induced further modulation of candidate genes, with *Ecc15* infection resulting in more numerous significant changes of expression. As such, some observed changes in candidate gene expression were specific to *Ecc15* infection, suggesting that bacterial species are able to induce differential responses within the *Drosophila* embryo with regards to the 20-HE signaling machinery.

Figure 3.21: Damage and Infection induce modulation of genes implicated in 20-HE signaling

(**A-C**) Comparison of relative levels of expression of candidate genes involved in 20-HE signaling in non-infected (NI) and PBS injected, and PBS and bacteria injected Stage 15 embryos (n=3, with ≥200 embryos per replicate). Genes involved in 20-HE biosynthesis and metabolism, as well as effector genes, are significantly downregulated upon damage induced via PBS treatment (**A**; p≥0.05, Hypothesis test). Further gene modulation is observed upon treatment with *Ecc15* (**B**) or *Mg1655 E. coli* **(C**), compared to that noted upon PBS injection. *Ecc15* specifically downregulates *eip71CD, mld phm* and *nvd,* whereas *Mg1655 E. coli* specifically upregulates *mld* expression. This can be confirmed by direct comparison of gene expression levels upon *Mg1655 E. coli* and *Ecc15* infection (**D**). Expression of *mld, ptth, phm, nvd* and *eip71CD* is significantly modulated in *Ecc15* infected embryos compared to those infected with *Mg1655 E. coli* (p≥0.05, Hypothesis test). * indicates a significant reduction from control levels with p ≤ 0.05. All other changes should be considered as non-significant.


A
NI vs. PBS
Expression ratio (AU)
2.0
1.0
0.5
dare
mld
ptth
phm
nvd
eo
ecd
eip 71CD
eip 78C
B
PBS vs. Ecc15
Expression ratio (AU)
4.0
2.0
1.0
0.5
0.25
0.125
dare
mld
ptth
phm
nvd
eo
ecd
eip 71CD
eip 78C
C
PBS vs. Mg1655
Expression ratio (AU)
2.0
1.0
0.5
dare
mld
ptth
phm
nvd
eo
ecd
eip 71CD
eip 78C
D
Ecc15 vs. Mg1655
Expression ratio (AU)
4.0
2.0
1.0
0.5
0.25
0.125
dare
mld
ptth
phm
nvd
eo
ecd
eip 71CD
eip 78C

3.3 Discussion

3.3.1 *Drosophila* embryos are able to mount a robust immune response to bacterial infection

The primary aim of these studies was to develop the *Drosophila* embryo as a model system to study bacterial infection *in vivo*; to validate the innate immune capacity of the embryo. Initial results via infection of a *Drc-GFP* reporter line suggested that the Stage 15 *Drosophila* embryo was able to mount a relatively robust innate immune response to both basic and more complex bacterial infections, such as *DH5α E. coli* and *Ecc15* respectively. This included the induction of AMP genes upon infection, localized to the epidermal cells at 6 hours post infection. As such, this data provides confirmation of the preliminary experiments conducted by Onfelt Tingvall *et al.* (2001), whereby epidermal expression of *Cec-lacZ* reporter was noted in late stage embryos upon infection with LPS or *E. cloceae B12*. However, not all microbial species tested induced a robust immune response within the Stage 15 embryo. Our results demonstrate that whilst Stage 15 embryos were able to drive a robust *Drc-GFP* expression in response to *E. coli sp.*, *Ecc15* and *P. luminescens* infection, injection of *S. typhimurium* did not induce a significant reporter response. *S. typhimurium* has previously been shown to be a strong inducer of the IMD pathway and the melanisation response in adult flies (Ayres and Schneider, 2008; Mabery *et al.,* 2010) and thus has been used extensively within infection studies in *Drosophila* models (Takeuchi *et al.,* 2000; Tzou *et al.*, 2000; Nagai *et al.,* 2002; Leulier *et al.,* 2003; Brandt *et al.,* 2004; Pham *et al.,* 2007; Schneider *et al.,* 2007; Ayres and Schneider, 2009; Jia *et al.,* 2009). However, this may not apply within the context of the embryo model, or else this result may be due to the initial dose of *S. typhimurium* administered; a bacterial suspension with a OD_{660} value of 1 was injected into Stage 15 embryos, whereas infection studies in adult flies have previously utilised values of 0.1 (Brandt *et al.,* 2004; Ayres and Schneider, 2008; Gordon *et al.,* 2008; Ayres and Schneider, 2009), hinting at the high pathogenicity of this bacterial species in *Drosophila* systems. Thus, it is possible that the dose of *S. typhimurium* administered to Stage 15 *Drc-GFP* embryos was excessive and

resulted in rapid embryo pathogenesis and mortality before an effective *Drc-GFP* response could be induced.

In terms of the levels of AMP gene induction observed within the embryo, changes upon infection were consistent with that observed in previous studies in a variety of *Drosophila* immune models. For instance, *AttA* was upregulated by 22.1-fold and *Def* by 6.7-fold at 4 hours post infection with *Ecc15* within the larval gut (Buchon *et al.,* 2009a). This is consistent with the relative values observed within the Stage 15 *Drosophila* embryo upon *Ecc15* infection: 9 and 30-fold for *AttA* and *Def* respectively, taking into account the effect of PBS injection on AMP gene induction. Studies in adult flies (Liehl *et al.,* 2006; Gendrin *et al.,* 2009) and larvae (Zaidman-Remy *et al.,* 2006), also provide comparable levels of upregulation of AMP gene expression to that observed within the *Drosophila* embryo model. Thus, this would infer that the embryonic AMP response is similarly robust to other *Drosophila* immunity models.

Moreover, other facets of the *Drosophila* immune system that have been characterized in adult fly and larval systems were also shown to be present and functional in the *Drosophila* embryo; further hinting at the completeness of the embryonic immune system. For instance, Stage 15 WT embryos were demonstrated to be able to phagocytose RFP-labelled *E. coli*; a process which was dependent upon SCAR, a member Arp2/3 complex responsible for actin nucleation. These data would suggest that the cellular branch of the *Drosophila* immune system is functional within the Stage 15 embryo, and moreover is relatively effective at mediating bacterial clearance, with approximately 40% of individual bacteria phagocytosed at only 1 hour post infection. The observed involvement of the Arp2/3 complex protein SCAR, an established regulator of actin nucleation (Machesky *et al.,* 1999; Welch and Mullins, 2002; Zallen *et al.,* 2002), for successful mediation of embryonic phagocytosis of *E. coli* and survival upon *Ecc15* infection is also consistent with the *in vitro* work of Pearson *et al.* (2002) and Rogers *et al.* (2003), who demonstrated that SCAR is required for internalization of *E. coli* and *S. aureus* particles by S2 cells respectively. Peltan *et al.* (2012) also provided evidence that SCAR was required for effective phagocytosis of the protozoan parasite

Leishmania donovani by mbn-2 cells. Thus, the embryonic cellular response is also governed by the same mechanistic principles of other *Drosophila* immune models, thus further demonstrating its potential as a valid immunity model. However, it could be argued that due to the defect in migration observed in $SCAR^{\Delta 37}$ hemocytes (Evans *et al.,* 2013) might impact on the phagocytic potential of these hemocytes; that they are unable to migrate effectively to the site of bacterial threat and thus are not able to clear the infection. Thus, in this case, the phagocytic defect observed could be an artifact of ineffective cell migration. However, considering the observation that bacteria are passively washed over hemocytes within the embryo (Vlisidou *et al.,* 2009) and as such phagocytosis is due to the opportunistic interaction of hemocyte and pathogen to a degree, this would somewhat eliminate dependence of cell migration for effective phagocytosis. Furthermore, the fact that *SCAR*-deficient hemocytes were observed to have multiple bacteria adhered to the cell surface would support the notion that *SCAR* mutant hemocytes have a phagocytic defect which is separate from cell migration.

In addition to these demonstrations of systemic and cellular functionality, the completeness of the embryonic immune model extends beyond the direct regulation of the systemic and phagocytic responses. For instance, expression of *mas* was demonstrated to be significantly downregulated upon *Ecc15* and *M. luteus* infection. *Mas* is a gene involved in the regulation of serine proteases; the presence of a serine protease domain within the C terminal region could imply that it is an antagonist of such enzymes, sequestering targets to regulate enzymatic activity (Kwon *et al.,* 2000; Huang *et al.,* 2000; Irving *et al.,* 2001). Thus, its significant downregulation at an early time-point post infection is consistent with a decrease in serine protease inhibition, so as to allow immune pathway activation. Moreover this particular finding also demonstrates that higher levels of immune regulation are functional within the Stage 15 embryo; further demonstrating the relative completeness of embryonic immune regulation. Additionally, *GADD45* expression was increased upon infection with *Ecc15*, albeit in an insignificant manner. This would be consistent with the results of Stramer *et al.* (2008), who demonstrated that damage inflicted upon embryos by sterile laser ablation and

excisional damage in murine repair models induced the expression of *GADD45*. The fact that *Ecc15* itself inflicts a significant degree of mechanical damage within the host gut upon oral infection that results in a damage response to induce AMP expression and stem cell activation (Buchon *et al.,* 2009a) further corroborates the notion that *Ecc15* induces a significant degree of damage signaling, which may potentially include activation of *GADD45*. Furthermore, these changes were accompanied by modulation in a third candidate gene, *ddc*, which plays a crucial role in the melanisation response (Tang, 2009). The fact that *M. luteus* induced greater, although insignificant, expression of *ddc* compared to that of *Ecc15* would suggest that this particular Gram-positive microbe may induce greater levels of melanisation within the embryonic system, although no obvious signs of melanisation were detected within the infected embryos themselves (data not shown). On the contrary, *M. luteus* has been exhibited to induce melanisation in the mosquito *Aedes egypti* (Hillyer *et al.,* 2003; Hillyer *et al.,* 2005) as well as in adult *Drosophila* (Ayres and Schneider, 2008). Thus, an increase in *ddc* expression upon *M. luteus* infection within the embryo is not surprising. However, given the non-significant nature of these latter results, further investigation is required to discern if these findings have a true biological basis. In particular, induction of *GADD45* may be greater with bacterial species that induce greater levels of mechanical damage and *ddc* with strains that have been shown to initiate the melanisation cascade in other *Drosophila* immunity models, such as *S. typhimurium* (Ayres and Schneider, 2008). However, it is interesting that all three of these candidate genes were significantly upregulated upon the damage induced via PBS injection. This would suggest that these genes are important in the immune response mediated within the embryos upon damage alone, and also confirms that the immune response to damage within the embryo is not limited to solely AMP induction but potentially spans a more diverse range of genes, as suggested by previous studies (Stramer *et al.,* 2008).

Therefore, it is clear that *Drosophila* embryos are able mount not a robust response to infection by a range of pathogens, encompassing the deployment of a diverse array of immune mechanisms which are consistent with the

immune responses of previously characterized *Drosophila* adult, larval and cell culture models. Moreover, this response is not solely specific to infection but can also be induced by damage alone, corroborating previous data that such danger signals can also induce an immune response in vivo. Thus, the Stage 15 *Drosophila* embryo can be considered as a viable immune model to study bacterial infection.

3.3.2 Stage 15 *Drosophila* embryos are able to effectively discriminate between different types of infection

As well the confirmation of ability of the Stage 15 embryo to induce an AMP response to bacterial infection, results also demonstrated that the Stage 15 embryonic immune system was able to mimic the ability of *Drosophila* adult and larval systems to effectively distinguish between Gram-negative and Gram-positive infections, and interestingly between different Gram-negative bacterial species. This was determined by monitoring the induction of different AMP gene classes upon *Mg1655 E. coli*, *Ecc15* and *M. luteus* infections in Stage 15 embryos. Similar trends were noted to those of Lemaitre *et al.* (1997), whereby Gram-negative AMPs were induced by infection with strains of *E. coli* and Gram-positive AMPs by *M. luteus*. Moreover, the observation that *Dpt* upregulation was disrupted in rel^{E20} mutant embryos, and *Drs* in $modSP^{1}$ mutants, post infection with *Ecc15* or *M. luteus* respectively, is an effective demonstration of not only the functionality of the embryonic IMD and Toll pathways but also that the embryonic immune system regulates AMP expression in a manner concordant with adult flies, larvae and *in vitro* immunity models (Lemaitre *et al.,* 1997; De Gregorio *et al.,* 2001; Irving *et al.,* 2001; Hoffmann, 2003; Brennan *et al.,* 2004; Lemaitre and Hoffmann, 2007; Flatt *et al.,* 2008; Buchon *et al.,* 2009; Valanne *et al.,* 2011). Thus, the embryonic AMP response is not aspecific, but utilizes both the IMD and Toll signaling pathways to generate disparate AMP responses to differential types of infection, as in other *Drosophila* immunity models.

However, evidence from the embryonic immune model may suggest that the sub-divisions of the AMP response in *Drosophila* may be relatively arbitrary, and that the reality of AMP gene induction upon infection may be more complex than previously perceived. For instance, *Ecc15*, a Gram-negative bacterium, was noted to stimulate a significant upregulation of *Def* and *Mtk*, key AMPs induced in response to Gram-positive microbes and fungal species respectively, whilst those embryos injected with *M. luteus* were surprisingly noted to lack any significant upregulation of *Def*. However, these observations are not unique to the *Drosophila* embryo; upregulation of *Def* upon Gram-negative bacterial infection has been well documented in other *Drosophila* immunity systems (Lemaitre *et al.,* 1997; Buchon *et al.,* 2009a), whilst there are also examples of its clear anti-Gram-positive activity (Rutschmann *et al.,* 2002; Tzou *et al.,* 2002). Similarly, *Mtk* has been noted to be highly induced upon infection with both Gram-negative and Gram-positive bacterial species, as well as during fungal infection (Levashina *et al.,* 1995; Lemaitre *et al.,* 1997). These phenomena could result from cross-talk between the IMD and Toll pathways, as suggested by Tanji *et al.* (2007). Moreover, other *in vivo* studies have noted that *Def* expression is not induced by infection of *M. luteus*; Nehme *et al.* (2011) noted that infection of adult flies with *M. luteus* did not induce *Def* expression, despite the documented antibacterial activity of *Def* against *M. luteus* observed in *in vitro* systems (Dimarcq *et al.,* 1994; Tzou *et al.,* 2002). These results, therefore, are not suggestive of anomalies within the embryonic immune response, but substantiate the true nature of AMP expression within *Drosophila*; that arbitrary subdivisions of AMPs into rigid classes may not always reflect the underlying biology of the system. Moreover, it also provides further confirmation that the embryonic immune response to bacterial infection closely mirrors that of other *Drosophila* immunity models.

A further interesting feature of the data was that disruption of either IMD or Toll signaling led to a significant decrease in AMP gene transcription upon bacterial stimuli, which was not significantly different to the level induced by PBS injection alone. However, considering that the damage induced by PBS injection was demonstrated to generate a lower level AMP response, it can be concluded that damage alone is able to induce an AMP response, but that

NFκB-related signaling is not required for this response. This, therefore, poses the question of what biological mechanism is regulating the damage-induced AMP response within the *Drosophila* embryo, independent of the IMD and Toll signaling cascades. One possible candidate may be the JAK/STAT pathway, which has been previously implicated in the response to wounding in larval wing discs, as well as after induction of cancerous tumours (Pastor-Pareja *et al.*, 2008). Another candidate may be the JNK pathway, as studies have shown that JNK activity is upregulated in response to damage inflicted in larval wing discs (Pastor-Pareja *et al.,* 2008), as well as demonstrating that JNK activity may be regulated by Tak1 (Boutros *et al.,* 2002, thus potentially linking IMD pathway signaling and JNK activity. To elucidate whether these pathways are required for the damage-induced AMP response in the Drosophila embryo, AMP gene transcription in embryos where either JAK/STAT or JNK signaling is disrupted could be assessed.

Interestingly, our data would also suggest that different species of pathogen are also able to induce more highly specific AMP responses within the embryo host. Whilst *Ecc15* and *Mg1655 E. coli* both greatly induced AMP expression, differences were observed between the relative levels of distinct AMP expression to infer that the embryonic host is able to differentiate between different bacterial species and adapt its AMP response accordingly. Whilst no studies have looked at this aspect of recognition in great detail, there are results within the scientific literature that would hint to corroborate this hypothesis. Results from Lemaitre *et al.* (1997) demonstrated that different Gram-negative species induced differential expression of a *Dpt-lacZ* construct, with species such as *P. aeruginosa* inducing greater expression than *Serratia marcescens*, although this study does not address the contribution of individual AMPs to the total host response. Similarly, Nehme *et al.* (2011) observed that differing levels of *Drs* expression were induced by three different bacterial species. Thus, in a preliminary manner, these results would support the hypothesis that recognition of bacterial species by the *Drosophila* immune system may be more highly specific than currently understood. Nevertheless, it is also clear that the *Drosophila* embryo system is able to recognize bacterial stimuli in a highly efficient manner and subsequently able to translate these

stimuli into appropriate immune responses. Taken together, these results would suggest that the recognition and signaling pathways of the embryonic immune system are as complete and effective as the larval and adult fly systems. Moreover, these results provide further confirmation that the *Drosophila* embryo immune system is dynamic; the fact that Stage 15 embryos can differentiate between two different species of Gram-negative bacteria is suggestive of a degree of immune plasticity within the embryonic AMP response; a surprisingly complex property for the immune system of a developing embryo to display.

3.3.3 The epidermis and trachea are primary sites of AMP production within the *Drosophila* embryo

Having established the robust immune potential of the Stage 15 embryo, a primary site of *Drc-GFP* expression was determined to be the embryonic epidermis, whereby individual epidermal cells displayed a loosely segmental pattern of reporter activation. As such, these observations are consistent with the results of Tingvall *et al*. (2001) and Onfelt Tingvall *et al.* (2001), whose authors also noted epidermal activation of AMP reporter constructs within the embryonic epidermis upon treatment with LPS. A more interesting finding was that the tracheal network was immune-responsive within the Stage 15 embryo, which has not been observed in previous studies of the embryonic immune capacity. However, the notion that the tracheal epithelia can mediate AMP expression is not novel; previous work by Tzou *et al.* (2000) using AMP reporter lines demonstrated that both *Drc* and *Drs* were expressed within the tracheae of *Drosophila* larvae and adults challenged with *Ecc15*, with *Drs* expression acknowledged to be under the control of IMD signaling. In a similar vein, transcriptomic studies have also elucidated that AMPs such as *Def*, *Mtk*, *Drs*, *AttA* and *Dpt* are expressed at a low basal level in trachea (Wagner *et al.,* 2008). The tracheal epithelium is also known to express a variety of pattern recognition receptors that are required for both Toll and IMD pathway functionality (Wagner *et al.,* 2008). Representative of the IMD pathway, a variety of PGRP isoforms that are required for activation and modulation of

pathway activity are present in tracheal tissue; these include PGRP-LE and PGRP-LC (Wagner *et al.,* 2008). Toll receptors, such as Toll, Toll-7 and Toll-8, have also been demonstrated to be present on the tracheal epithelia, alongside PGRP-SA and GNBP-1 and -3 (Wagner *et al.,* 2008), suggesting the potential for activation of both Toll and IMD signaling within this tissue. Thus, given the extensive presence of the systemic immunity recognition machinery within the trachea, it is not surprising that *Drc-GFP* expression was noted upon infection within the developing *Drosophila* embryo. As such, the response within the trachea of the Stage 15 embryo parallels that of both adult and larval systems.

Furthermore, the fact that expression of the *Drc-GFP* reporter construct in the Stage 15 embryo appeared to be relatively consistent between primary, secondary and terminal tracheal branches is concordant with results from Wagner *et al.* (2008), whose authors noted that all airway epithelial cells in 3rd instar larvae were relatively homogenous in their expression of a *Drs-GFP* reporter construct upon infection with either *Pseudomonas aeruginosa* or *Ecc15*, irrespective of their position within the tracheal network and the initial site of infection. This activation of the entire tracheal network, as seen in Stage 15 embryos upon damage and infection with *Ecc15*, could be due to a spread of activation of IMD signaling along tracheal branches in a non-cell autonomous fashion, as observed in larval models of infection (Akhouayri *et al.,* 2011); originating in the anterior of the embryo and spreading to the entire tracheal network in a global response. However, given that the tracheal network does not become functional for respiration until the end of embryogenesis, when the 1st instar larva emerges (Manning and Krasnow, 1993; Tsarouhas *et al.,* 2007; Ghabrial *et al.,* 2011), it is interesting to speculate as to what advantage AMP expression in the trachea above other tissues may provide. It could be speculated that as the tracheal network does not perform any respiratory function until the end of embryogenesis, that tracheal cells are less constrained in terms of energy or resources and thus are able to mount an immune response, in comparison to other immune competent tissues which may be required to perform other functions in addition to their role in defense. In this sense, it may be advantageous for the

embryo to mount an AMP response from a tissue which does not carry this burden.

Whilst the epidermal and tracheal *Drc-GFP* response to bacterial stimuli was extensive in terms of coverage and resulted in intense activation of the reporter in individual epidermal cells, no activation of *Drc-GFP* was observed within the developing fat body, consistent with the findings of Onfelt Tingvall *et al.* (2001) and Tingvall *et al.* (2001) whose work suggested that AMP responses by the fat body appeared to predominate once reaching first instar larval development whilst being absent from *Drosophila* embryos upon bacterial infection. This in turn poses the question of whether the response in the Stage 15 embryo is truly a systemic immune response, or whether its origins are more closely rooted in a collection of individual yet extensive local immune responses, regulated disparately or in concert. Nonetheless, other data would suggest that a traditional systemic immune response, with signaling mediated as observed in the fat body, is still required in *Drosophila* embryos. For instance, $modSP^{1}$ and $psh^{1};;modSP^{1}$ mutant Stage 15 embryos demonstrated a selectively increased susceptibility to injection with *M. luteus* and *A. oryzae* proteases, as well as significantly reduced *Drs* expression levels upon *M. luteus* infection, compared to WT or rel^{E20} mutant embryos, suggesting involvement of the Toll pathway within the embryonic immune response. In contrast, the local AMP responses observed in larval models are mediated solely via the IMD pathway (Tzou *et al.,* 2000), with no Toll pathway involvement as of yet recorded, even in the expression of Gram-positive or fungal AMPs, such as *Drs* (Ferrandon *et al.,* 1998). Supporting this assertion is the fact that not all components required for Toll pathway functionality are expressed within immune-responsive tissues, such as the tracheal network; whilst spz, Toll, MyD88 and the transcription factors Dif and Dorsal are expressed in the airway epithelium, the expression of other essential pathway components, such as the adaptor protein Tube and Pelle kinase, is absent (Wagner *et al.,* 2008), implying a lack of Toll pathway function in this tissue. In contrast, all IMD signaling members required for proper pathway functionality were shown to be expressed in the trachea epithelia (Wagner *et al.,* 2008), indicative of a preference for IMD signaling in the local AMP response within

this tissue. Therefore, if the AMP response in the Stage 15 embryo is truly local in nature, this data may represent the first indication of Toll involvement in the local immune response. Alternatively, it may be that observation of other AMP reporter lines post-infection, aside from the *Drc-GFP* reporter line used in this study or the *Cec-lacZ* line employed by Tingvall *et al*. (2001) and Onfelt Tingvall *et al.* (2001) may elucidate an AMP response by the fat body to further clarify these results.

Moreover, questions concerning how the individual epidermal and tracheal AMP responses are mediated *in vivo* remain; for instance, whether these responses are regulated individually or in concert via an as of yet unidentified mechanism. Evidence of tracheal communication to other immune responsive tissues, and the resulting impact of this interaction on AMP expression, is relatively scarce and the pertinent studies do not provide much information about the biological mechanisms behind these phenomena. One study has demonstrated that inactivation of Serpin77Ba, which under basal conditions inhibits melanisation in tracheal tissue, can induce expression of *Drs* via the Toll pathway in the fat body of 3rd instar larvae (Tang *et al.,* 2008), inferring the ability of the trachea to not solely mediate the spread of innate immune responses within its own cells, but also to facilitate changes in the immune-responsiveness of other tissues to adapt to the immune challenge deployed. One could speculate that terminal tracheal branches themselves may be responsible for communicating the requirement for an AMP response to epidermal cells; further propagating the embryonic immune response. It has been well established that there is extensive communication between epidermal cells and the tracheal network during embryogenesis; permitting correct tracheae morphogenesis and growth of tracheal tubes. For instance, primary tracheal branches themselves originate from placodes in the epidermal ectoderm (Pereanu, 2006). Furthermore, growth of the primary and some secondary tracheal tubes is induced by the strategic expression of the FGF homologue Bnl in epidermal cells (Pereanu, 2006). Thus, given the close spatial and developmental relationship between the epidermis and tracheal network, as well as the ability of the tracheal system to propagate AMP responses to neighbouring cells within its own network (Wagner *et al.,* 2008),

the potential for communication of the innate immune responses upon the urgency of infection is relatively plausible. The propagation of the *Drc-GFP* response by the trachea would also potentially explain the earlier activation of the *Drc-GFP* construct in trachea compared to the epidermis, as well as the apparent segmental nature of epidermal *Drc-GFP* expression, as the pattern of tracheal branches is itself segmental and highly conserved between individuals (Klambt *et al.,* 1992; Wilk, 1996; Chung, 2011). Whilst speculative, confirmation of this hypothesis would highlight the importance of the trachea as an immune-responsive tissue within the *Drosophila* embryo. However, further work is required to elucidate if this is the case, and by what biological mechanism this phenomenon could potentially occur. The precise role of the trachea in inciting the expression of AMPs within epidermal tissue could be elucidated via monitoring *Drc-GFP* expression post infection within embryos in which trachea function is disrupted, such as those carrying a mutation in the *breathless* (*btl*) or *trachealess* (*trchl*) genes; to determine if the trachea is required for epidermal *Drc-GFP* expression. As such, the *Drosophila* embryo could be a valuable model system for investigating how AMP responses by individual tissues are mediated.

3.3.4 20-HE is required to switch on immune competence within the *Drosophila* embryo

Whilst *Drosophila* embryos at Stage 15 of development were demonstrated to be robustly immune competent, those at Stage 11 of embryogenesis were demonstrated to be immunologically naïve; starkly lacking the AMP response exhibited by Stage 15 embryos post infection with bacterial species, resulting in increased mortality. The immune incompetence of Stage 11 embryos could be rescued by treatment with 20-HE, mimicking the 20-HE pulse observed during embryogenesis. Conversely, the disruption of 20-HE signaling within Stage 15 embryos rendered them immune incompetent; an increased susceptibility to *Ecc15* infection and complete inhibition of a subset of AMP genes was noted. Thus, this data suggested that 20-HE facilitates the maturation of the embryonic immune system. These *in vivo* observations are supported by previous in vitro studies using S2 cells. These include the

observation that S2 cell expression of AMP genes upon stimulation with PGN can be enhanced via pre-treatment with 20-HE (Flatt *et al.,* 2008). Moreover, the demonstration by Rus *et al.* (2013) that the expression of PGRP-LC, a major receptor for Gram-negative PGN that initiates IMD signaling (Choe *et al.,* 2002; Gottar *et al.,* 2002; Leulier *et al.* 2003; Kaneko *et al.,* 2004; Takehana *et al.,* 2004; Kaneko *et al.,* 2006), is regulated by 20-HE in S2 cell cultures, as well as the evidence indicating 20-HE involvement in an antagonistic relationship with JH to modulate the innate immunity of S2 cells (Flatt *et al.,* 2008), further supports the potential for the pulse of 20-HE during mid-embryogenesis to act as a regulator of immune system maturation in *Drosophila* embryos. However, the rescue of Stage 11 embryonic immune competence was not complete; the percentage of Stage 11 embryos demonstrating *Drc-GFP* expression remained highly significantly different to that of Stage 15 controls. This could be due to the rather crude nature of the assay; co-injecting 20-HE and *Ecc15* did not wholly resemble the natural 20-HE pulse at Stage 12 and the subsequent infection of bacteria at Stage 15 of development that had previously been utilized. As such, an approach whereby priming of the Stage 11 embryos using 20-HE and consequent infection at Stage 15 may have produced higher levels of response.

Furthermore, manipulation of 20-HE signaling in *Drosophila* embryos also resulted in observations that do not fit within the current paradigm. One result which was somewhat contrasting to *in vitro* work of Rus *et al.* (2013) was the expression of AMP genes in Stage 15 EcR^{Q50st} homozygous mutants. Whilst the upregulation of *Cec, Mtk* and *Def* gene expression was effectively eliminated in Stage 15 EcR^{Q50st} embryos upon *Ecc15* infection compared to the relatively robust response normally observed in WT embryos, no difference was observed in *Dpt* expression between WT and EcR^{Q50st} counterparts. Moreover, *Drc* and *AttA* expression was significantly increased in EcR^{Q50st} mutants compared to WT. This is somewhat in contrast to the in vitro results of Rus *et al*. (2013), who exhibited that *Dpt, Mtk* and *Drs* expression upon PGN stimulation in S2 cells required 20-HE pretreatment, even upon overexpression of PGRP-LC. Moreover, *Cec, AttA* and *Def* expression in S2 cultures upon challenge was demonstrated to not strictly

require 20-HE pre-treatment, only overexpression of *PGRP-LC* (Rus *et al.,* 2013). However, there is some overlap of results from these S2 screens and from the Stage 15 *Drosophila* embryo. For instance, expression of *Mtk* is severely inhibited in both the Stage 15 EcR^{Q50st} mutant embryo and in S2 cells not pre-treated with 20-HE. The fact that this result demonstrated the greatest significant difference between EcR^{Q50st} mutant and WT embryos would also further highlight the relevance of these results. However, the majority of the results from the *in vitro* S2 cell screens of Rus *et al.* (2013) are not concordant with the results from infection studies with Stage 15 EcR^{Q50st} mutant embryos, suggesting that 20-HE regulation of innate immunity may vary between *in vitro* and *in vivo* systems. However, the results attained from EcR^{Q50st} mutant embryos also differ from other *in vivo* studies. Adult flies carrying mutations in the EcR ligand-binding domain or in specific EcR introns were shown to have a significantly decreased expression of *Dpt* upon *E. coli* infection (Rus *et al.* 2013), thus suggesting that significant differences may exist between the 20-HE regulation of immunity at different stages of development.

Of particular interest is the observation that expression of *AttA* and *Drc* in EcR^{Q50st} mutant embryos was not only induced upon *Ecc15* infection, and moreover to a level that was significantly higher than that observed in WT controls. This is somewhat in agreement with in vitro studies conducted by Rus *et al.* (2013) using S2 cell cultures, where it was witnessed that *AttA* expression did not necessarily require 20-HE pre-treatment of cultures; in this case, ectopic expression of *PGRP-LC* was sufficient to bypass the requirement for 20-HE to render S2 cells immune competent. It is difficult to assess if the increase of *Drc* expression witnessed in Stage 15 EcR^{Q50st} embryos upon *Ecc15* infection compared to WT counterparts has any basis in vitro, as the expression of this AMP was not investigated by Rus *et al.* (2013). Another potential explanation for these rather anomalous results may lie in the nature of the EcR^{Q50st} mutation. This mutation selectively incapacitates an exon of the EcR-B1 isoform, leaving the functionality of the A and B2 isoforms intact. It may be possible that some redundancy exists between the isoforms, or some transcriptional division permitting the transcription of a subset of AMP genes, such as *Dpt, Drc* and *AttA*; permitting increased expression of the AMP

subset to attempt to compensate for the loss of expression of other AMPs. The concept of differential mechanisms to regulate individual AMP genes is not novel. Rus *et al.* (2013) identified two sub-divisions of AMP genes; those that required 20-HE signaling and those in which this requirement could be bypassed by other mechanisms. Similar divisions may exist within the embryo, and inactivating all isoforms of the EcR may allow these to become more apparent. However, the fact that basal levels of *Dpt, AttA* and *Drc* were significantly lower than those observed in WT counterparts is suggestive of a compromise in innate immunity even prior to an infective state, which may impact on the long-term survival of EcR^{Q50st} embryos, as indicated in the viability of mutant embryos post infection with *Ecc15*. Although the exact nature of this compromise remains elusive, these collective results imply that 20-HE regulation of innate immunity may in fact be far more complex than the current paradigm suggests.

Nevertheless, these results also give an important insight into the importance and contributions of individual AMP genes to the embryonic immune response. The fact that Stage 15 EcR^{Q50st} mutant embryos are highly susceptible to infection with *Ecc15*, with viability post infection reduced to levels normally observed with immune-compromised embryos, yet are able to greatly induce the expression of both *Drc* and *AttA* would suggest that these AMPs are not crucial to the embryonic immune response. This also consistent with the work of Tzou *et al.* (2002), who presented data to suggest that overexpression of a single Gram-negative AMP gene cannot completely compensate for loss of function in IMD/Toll double mutants; hence, a more collective AMP response may be required to completely rescue viability of EcR^{Q50st} embryos. Alternatively, the biological reason for the dispensable nature of Drc and AttA within the embryonic immune response may be clarified by considering their antimicrobial mode of action. For *Drc*, this involves the inactivation of the intracellular heat shock protein DnaK, which ultimately leads to disruption of bacterial metabolism and cell death (Bikker *et al.,* 2006). As such, the timescale of *Drc* action and effectiveness against bacterial foes may be lengthier than that of other AMPs. As such, *Drc* may play a role in later stages of the host response, and its initial involvement for controlling bacterial load

relatively limited. Hence, it could be relatively dispensable in the embryonic immune response to *Ecc15* infection, where fast antimicrobial action to respond to bacterial kinetics may be vital. In a similar vein, attacins extracted from the giant silk moth *Hyalophora cecropia* were demonstrated to be more efficacious in actively dividing cells, with 10-50% of *E. coli* remaining after prolonged exposure to high levels of attacins in *in vitro* scenarios (Engstrom *et al.,* 1984). Thus, from the perspective of the embryo host, the deployment of *AttA* is relatively risky; investing in innate immune mechanisms, such as AMP production, is relatively costly (Yixin *et al.,* 2009) and *AttA* would only be effective after bacteria had begun to actively divide and potentially would not eradicate a large proportion of the bacterial threat. As such, it may be that the relatively low proportion of *AttA* induced by Stage 15 embryos upon *Ecc15* infection may reflect this concept. On the other hand, the inhibition of *CecA1, Mtk,* and *Def,* which could potentially contribute to the high mortality rate of EcR^{Q50st} embryos, would suggest that these AMPs play a greater role in the host response to Ecc15 infection. Interestingly, *CecA1* and *Mtk* genes are amongst a cluster of immune genes that are acutely induced at early time points after infection (Boutros *et al.,* 2002) with *in vitro* screens of *CecA* antibacterial activity elucidating that minimal inhibitory concentrations of this AMP can kill all bacteria encountered within 5 minutes of contact. Thus, it may be that a rapid response to *Ecc15* infection is required within the Stage 15 embryo to control both bacterial load and deleterious host-pathogen interactions. Thus, taking the antibacterial properties of individual AMPs into account and given that Stage 15 embryos infected with *Ecc15* rely on *CecA1* as a large proportion of their AMP response, supported by more moderate proportions of *Def* and *Mtk*, it is not particularly surprising that a deficiency in these three AMPs may prove fatal for EcR^{Q50st} mutant embryos upon infection.

Of further interest is the location for the requirement of 20-HE signaling and thus immune competence in *Drosophila* embryos to ensure immune competence. Expression of a dominant-negative form of the EcR-B1 receptor was driven in either hemocytes or the trachea and the susceptibility of the resulting Stage 15 embryos to *Ecc15* infection observed; to determine if inhibition of 20-HE signaling in either of these tissues was required for

effective embryonic survival to infection. The observation that Stage 15 embryos expression the EcR-B1 dominant negative construct in hemocytes (*srp>EcR-B1 DN*) did not exhibit a significantly different viability post infection with *Ecc15* compared to WT controls suggests that these immune cells may not play a significant role within the maturation of the embryonic immune system. This finding almost seems counterintuitive, considering that S2 cells, which display many characteristics of macrophage-like cells, require 20-HE application in order to render them immune-competent (Flatt *et al.,* 2008; Rus *et al.,* 2013) and the proposed synergistic relationship between the systemic and cellular branches of the *Drosophila* immune response (Agaisse *et al.,* 2003; Charroux and Royet, 2009).

More interesting is the observation that EcR-B1 is required in the trachea for effective resistance to *Ecc15* infection, as determined by the reduced viability of Stage 15 embryos expressing EcR-B1 dominant-negative protein under the control of the breathless promoter (*btl>EcR-B1 DN*) as well as the increased bacterial load noted in Stage 15 *btl>EcR-B1 DN* embryos. Aforementioned results have demonstrated that the trachea is a major immune-responsive responsive tissue within the Stage 15 embryo upon *Ecc15* infection, as well as playing a role in the local immune response in both larval and adult fly models (Tzou *et al.,* 2000). Thus, there appears to be a close relationship between the requirement of 20-HE signaling and the immune capacity of tissues within the *Drosophila* embryo, despite the ubiquitous nature of the EcR. Further investigation could focus on the transcriptome of *btl>EcR-B1 DN* embryos; to determine if the expression levels of specific AMP and other immune candidate genes are concordant with those observed in the EcR^{Q50st} mutant. This in turn would facilitate a greater understanding of the precise role of the trachea in the maturation of the embryonic immune response. Given the role of epidermal cells in directing the growth and development of the embryonic tracheal network, it would be interesting to determine if there is any direct interaction between tracheal and epidermal tissue in the immune maturation of the tracheal network. Furthermore, it has been postulated that the pulse of 20-HE during mid-embryogenesis may arise from epidermal cells, as opposed to the ring gland which is not fully functional until late stages of embryogenesis

(Warren *et al.,* 2002), as determined by the expression of 20-HE synthesis genes, such as *dib* and *sad*. Thus, it would be of interest to assess the contribution of the epidermis itself to the maturation of the embryonic immune system.

3.3.5. *Ecc15* infection modifies expression of 20-HE signaling components

These results open up more general questions of the precise mechanism of *Ecc15* killing of the *Drosophila* embryo. Even relatively immune robust Stage 15 embryos showed significantly decreased viability to low doses of *Ecc15* compared to both controls and counterparts infected with other species, such as *E. coli*, demonstrating the relatively toxic nature of *Ecc15* to the *Drosophila* embryo. This is in contrast to other *Drosophila* models, whereby *Ecc15* infection does not cause significant mortality (Buchon *et al.,* 2009a). Whilst the majority of our studies have been focused on the host response to eradicate invading pathogens, it is important to acknowledge that infection is a two-way interaction. Hence, it has been acknowledged that, whilst the host may deploy a diverse range of responses to eliminate the threat by the pathogen, the invading infective agents are also able to subvert the host's immune response machinery for its own benefit to further sustain infection (Jones *et al.,* 2012; Baxt *et al.,* 2013; Lutay *et al.,* 2013; Rolando *et al.,* 2013). Thus, it is a logical assumption that if the 20-HE signaling confers a great immune advantage on the host, then it is also probable that bacteria may have developed mechanisms to manipulate this signaling pathway, to the detriment of the host. The above data demonstrating the modulation of 20-HE synthesis and effector gene expression may be confirmation of this hypothesis. For instance, selective downregulation of genes involved in ecdysone synthesis upon *Ecc15* infection, such as that of *mld*, may represent manipulation of the host 20-HE signaling machinery to ultimately downregulate expression of AMPs. Downregulation of expression of other candidate genes upon *Ecc15* infection, such as the 20-HE signaling effector *eip71CD*, could also indicate that this subversive effect on the embryonic immune response could extend beyond the expression of AMPs. Eip71CD has been shown to play roles in both the

oxidative stress response and the autophagic response to cell death (Sun *et al.,* 1999; Gorski *et al.,* 2003; Weissbach *et al.,* 2005). Nevertheless, the question remains as to how *Ecc15* is able to modulate host gene expression; the precise mechanism by which this proposed subversion occurs. It has been well established that bacterial species are able to modify host gene expression via inducing changes in host DNA topography. For example, *Legionella pneumophila* has been revealed to secrete a virulence factor, RomA, which translocates to the infected host cell nucleus and promote a methylation burst, dramatically changing the host chromatin landscape and permitting the repression of host gene expression (Rolando *et al.,* 2013). Moreover, virulent *E. coli* strains were proven to interfere with RNA polymerase II activity, leading to suppression of the host disease-associated response (Lutay *et al.,* 2013). Thus it is entirely possible that the manipulation of the embryo's own 20-HE signaling could contribute to the mortality observed within Stage 15 embryos upon *Ecc15* infection, although the mechanism by which this occurs remains an open question.

It is interesting that many of the changes in 20-HE biosynthesis gene expression observed were specific to *Ecc15* infection, as injection of *Mg1655 E. coli* within Stage 15 embryos induced disparate effects on gene expression. For instance, *mld* expression was upregulated upon infection with *Mg1655 E. coli*, whereas expression levels were dramatically downregulated upon *Ecc15* infection in Stage 15 embryos. Furthermore, the downregulation of *eip71CD, phm* and *nvd* expression observed with *Ecc15* infection was not observed following *Mg1655 E. coli* infection in Stage 15 embryos. This not only confirms the potentially more pathogenic nature of *Ecc15* to Stage 15 *Drosophila* embryos, but also demonstrates that different bacterial species can modify host gene expression to differing degrees and thus some of the effects on host gene expression exerted by *Ecc15* may be species specific. As such, the embryo system has aided the identification of candidate genes which may hold the key to gaining further knowledge about *Ecc15*-host dynamics.

Nevertheless, some genes involved in 20-HE biosynthesis, such as *ptth,* exhibited upregulation upon infection with *Ecc15*, which is not concordant with

the above hypothesis of *Ecc15* subversion of the host 20-HE signaling machinery. This is also the case with the observed downregulation of the 20-HE metabolism gene *eo*, indicating that 20-HE should theoretically persist for longer within the embryo system. It is possible that these are the true embryonic host responses to *Ecc15* infection, to increase 20-HE concentrations to enhance the embryonic immune response, and for which invading *Ecc15* bacteria are yet to adapt a suitable subversive response. However, it is difficult to draw any reliable conclusions regarding this conjecture, as it is difficult to separate the true host transcriptional response from those responses that arise due to pathogen subversion due to the nature of the data.

Also of note is the data which discloses the apparent decrease in expression of 20-HE synthesis, metabolism and effector genes upon damage induced by PBS injection of Stage 15 embryos alone, at levels intermediate between those observed in non-injected and infected embryos. This encompasses decreased expression of *ptth, phm, nvd* and *ecd* in terms of 20-HE synthesis genes, and the effector gene *eip78C*. This may reflect the downregulation of 20-HE synthesis to prevent chronic immune stimulation, which is known to be deleterious to the host (Janeway and Medzhitov, 2002; Cario, 2008; Medzhitov, 2008; Kambris *et al.,* 2009). If correct, this hypothesis would also imply that damage and repair responses may also be regulated via 20-HE signaling within the embryo. Further experiments to test this concept may involve investigating the involvement of 20-HE in the repair of wounds generated by sterile laser ablation or the recruitment of hemocytes to such wounds.

3.4 Conclusions

To conclude, the Stage 15 *Drosophila* embryo is able to mount a relatively robust immune response to bacterial infection. This includes the induction of AMP genes upon a range of bacterial stimuli; a response which is able to effectively discriminate between differential types of bacterial infection, as well

as individual bacterial species. This response is mediated via the characterized *Drosophila* systemic immunity pathways and localized to the epidermis and trachea. The cellular branch of the *Drosophila* immune response is also functional within the Stage 15 embryo, and appears to be under the regulation of mechanisms that are concordant with those observe in other *Drosophila* immunity systems. Moreover the modulation of other immune candidate genes would suggest that the Stage 15 embryonic immune system is relatively complete and comparable to other *Drosophila* immunity models. Immune competence arises at approximately mid-embryogenesis, under the control of the 20-HE pulse, as demonstrated by the partial rescue of AMP expression and bacterial clearance in Stage 11 embryos upon 20-HE co-administration with infective agents, although this does not confer any ultimate benefit upon survival to *Ecc15* infection itself. This may be due to the manipulation of the host 20-HE signaling machinery by *Ecc15* itself.

Chapter 4: Analysis of the Stage 15 *Drosophila* Embryo Global and Hemocyte Transcriptional Responses to Infection and Damage

4.1 Introduction

4.1.1 *Drosophila* model systems to investigate global transcriptional changes upon infection

Transcriptional profiling via microarray studies has revolutionized molecular biology over the past decade; permitting research efforts to move away from the more limited study of a few related genes, to the more holistic study of global cellular activity via the ability to monitor the abundance of thousands of transcripts. Hence, the power of microarray technologies has found application in numerous research areas, including the study of host-pathogen interactions. In fact, Jenner and Young (2006) acknowledge that this technology has greatly advanced the understanding of host-pathogen interactions as it has permitted the study of the reprogramming of the host transcriptome during infection, a central component of the host defense. This is reflected in the vast numbers of publications focused on host transcriptional profiling; Jenner and Young (2006) cite that in the first 6 years alone after the advent of microarray technology, more than 160 papers detailing transcriptional profiling experiments were published, involving 25 different host species, 26 different bacterial species, 30 viral species, yeast and helminth species; demonstrating the power of transcriptional profiling to elucidate vast and valuable novel information concerning the host-pathogen interaction. As such, transcriptional profiling approaches have been employed in a variety of insect model systems to study the global transcriptional response to a variety of infective agents, including bacterial, fungal and parasitic infections. These include the elucidation of the transcriptional profile of the mosquito *Aedes aegypti* upon infection with the dengue virus (Sim and Dimopoulos, 2010), the comparison of transcriptional responses driven by human and rodent species of the *Plasmodium* parasite within the mosquito *Anopheles gambiae* (Dong *et al.,* 2006) and the study of

the genes transcribed upon injury in the diamondback moth *Plutella xylostella* (Eum e*t al.,* 2007).

Given the prominence of *Drosophila* as an immunity model, its application in such microarray studies has been extensive, with strategies to examine the widespread transcriptional changes upon infection in *Drosophila* encompassing both *in vitro* and *in vivo* systems. For instance, the transcriptional profile of cultured S2 cells upon *E. coli* infection permitted the identification of three novel AMP gene regulators and the initial elucidation of JNK as a regulator of AMP gene expression (Kallio *et al.,* 2005). Further microarray studies utilized the *Drosophila* in vitro systems to characterize the transcriptional responses of S2 cells to specific yet diverse pathogens, including *Wolbacchia* (Xi *et al.,* 2008), *C. albicans* (Levitin *et al.,* 2007) and the *Drosophila* C virus (Zhu *et al.,* 2013). Microarray studies have also found wide application in the study of the transcriptional response to infection by in vivo *Drosophila* systems. For instance, De Gregorio *et al.* (2001) used high-density oligonucleotide microarrays to study genome-wide transcriptional responses of *Drosophila* adults to *E. coli, M. luteus* or *Beauvaria bassiana* stimuli. Similar microarray studies were also performed by Irving *et al.* (2001), whose authors used male adult flies to assess the genome-wide transcriptional responses to Gram-negative, Gram-positive and fungal pathogens. Further microarray strategies employed in *Drosophila* adult flies involved the comparison of transcriptional profiles of Toll and IMD mutants at different time-points post infection with a range of bacterial and fungal pathogens, including *E. coli, M. luteus* and *E. faecalis* and *A. fumigatus* and *B. bassiana* respectively (De Gregorio *et al.,* 2002). Conversely, Vodovar *et al.* (2005) employed *Drosophila* 3rd instar larvae in microarray studies to elucidate the transcriptional profile generated upon *Pseudomonas entomophila* infection and Wertheim *et al.* (2005) generated the transcriptional profiles of 2nd instar larvae infected with the parasitoid *Asobara tabida*. Nonetheless, whilst there is a plethora of studies elucidating the organism-wide transcriptional responses to infection, other *Drosophila* microarray studies have concentrated solely on the responses of specific tissue types to invading microorganisms; to attempt to spatially resolve the *Drosophila* immune response. For instance, Buchon *et al.*

(2009a) examined the genes specifically induced in dissected adult guts upon oral infection with *Ecc15*, subsequently demonstrating that immune responses in the gut were driven via JAK-STAT and IMD signaling.

However, the application of microarray techniques has not been limited to investigating solely infection-related transcriptional changes in *Drosophila*; recent studies have also used cDNA microarrays to attempt to dissect the individual responses of the host to infection, stress and damage. The microarray studies of Buchon *et al.* (2009a) revealed that the immune response of the *Drosophila* gut upon oral infection with *Ecc15* is mediated by IMD and JAK-STAT signaling, but also included the modulation of stress response gene expression and an increase in stem cell proliferation. Validation experiments further suggested that gut homeostasis is maintained via a balance of cell damage due to bacterial killing and repair of the epithelium by stem cell division (Buchon *et al.,* 2009a). Furthermore, microarray studies examining the transcriptional profiles of flies deficient in Mekk1, a MAPK kinase kinase (MAPKK) implicated in environmental stress responses (Inoue *et al.*, 2001), determined that Mekk1 regulated *Tot* stress gene expression upon septic injury (Brun *et al.,* 2006), permitting study of the interplay between stress and immune responses.

However, despite this apparent wealth of knowledge concerning the transcriptional profiles of *Drosophila* model systems upon infection and damage, very little work has been performed to elucidate the global transcriptional response of the *Drosophila* embryo under either of these scenarios. The only published account of the embryonic transcriptional profile upon damage stimuli is that of Stramer *et al.* (2008), who compared the transcriptional profiles of wounded and unwounded wild-type and hemocyte-null Stage 15 *Drosophila* embryos to attempt to identify novel wound- and hemocyte-specific genes, as well as to evaluate the ability of mechanical damage to induce an innate immune response. This approach permitted the identification of *GADD45* as a novel inflammation-associated gene, which was subsequently validated in murine models, as well as facilitating the observation that sterile damage is able to trigger induction of AMP genes

(Stramer *et al.,* 2008). Nevertheless, the nature of the embryonic global transcriptional responses to different types of infection, and how these responses differ to that generated as a result of purely damage stimuli, remains elusive.

4.1.2 Transcriptional profiling of hemocytes upon bacterial challenge and damage stimuli

Moreover, despite the diverse array of genome-wide transcriptional studies that have been performed on *Drosophila* models, relatively few have fully addressed the nature of the hemocyte transcriptional response to infection or damage. Of these, most have utilized immortalized cell lines that exhibit hemocyte-like characteristics, such as the S2 or mbn-2 cell lines, as an inference of actual hemocyte transcriptional behavior. For instance, microarray studies using mbn-2 cells performed by Johansson *et al.* (2005) investigated the disparate transcriptional profiles resulting from crude LPS or live *E. coli* infection. It was noted that only challenge with *E. coli* was able to induce significant expression of specific sub-sets of genes involved in tissue remodeling and stress signaling, whereas induction of AMP gene expression was stimulated by both LPS and live bacterial infection (Johansson *et al.,* 2005). Johansson *et al.* (2005) thus postulated that hemocytes were able to sense and differentially respond to purified bacterial surface molecules and infection with actively growing bacteria. However, it must be acknowledged that while these *in vitro* studies provide valuable inferences about hemocyte transcriptional responses, they are limited in the sense that such studies cannot account for the potential interactions that hemocytes may experience with other immune competent tissues upon infection *in vivo.* Nevertheless, as Pinto *et al.* (2009) note, it has previously been difficult assess the transcriptional profiles of these cells in a true *in vivo* context due to their relative scarcity in the circulating hemolymph, their adhesion to multiple tissues within the organism and the paucity of effective primary culture methodologies to generate sufficient material for genome-wide microarray studies. Moreover, looking to other insect models of immunity, this does not

appear to be a technical problem that is specific to *Drosophila*, as there are relatively few genome-wide microarray studies assessing the transcriptional contribution of hemocytes to the total host response in the *A. gambiae* (Pinto *et al.,* 2009) and *A. aegypti* (Choi *et al.,* 2012) mosquitos and the army worm *Spodoptera frugiperda* (Barat-Houari *et al.,* 2006). It must also be noted that even many mammalian macrophage infection transcriptional profiling studies rely on exposing macrophage-like cell lines, such as the mouse macrophage-like lines RAW264.7 (Eskra *et al.,* 2003) and J774A.1 (Andersson *et al.,* 2006) to bacterial species, as opposed to utilizing isolated primary cells. This would suggest that the problems associated with undertaking such experiments are widespread.

Stramer *et al.* (2008) used an alterative strategy to address this issue, by comparing the transcriptional profiles of wild-type and hemocyte-null *Drosophila* embryos upon damage inflicted via sterile laser ablation; to infer the existence of hemocyte-specific wound response genes *in vivo* whilst dispensing of the need to harvest hemocytes from live organisms. This approach permitted the assessment of the hemocyte contribution to the total unwounded embryo transcriptome and the subsequent discovery of wound-activated hemocyte genes, such as *phospholipase A1* and *Drs* (Stramer *et al.,* 2008). However, even this strategy yielded relatively few significantly upregulated or downregulated gene candidates for further study, possibly due to the effects of dilution of wound-induced genes. Thus, gaining a comprehensive view of the transcriptional changes that occur specifically within *Drosophila* hemocytes upon infection and damage remains technically challenging.

4.1.3 Strategies and Statistical considerations in the analysis of microarray data

In practice, a combination of strategies enable the effective management of the vast quantity of microarray data and permit the identification of candidate genes for further study. One such strategy is to assign terms to clusters of

gene that demonstrate similar functional attributes. Gene Ontology (GO) enables the classification of genes by molecular function, biological process or cellular component (Ashburner *et al.,* 2000; Baehrecke *et al.,* 2004), subsequently permitting a more functional overview of microarray data. Consequently, by combining the qualitative GO attributes and quantitative RNA level for microarray experiments, candidate genes can be identified without losing the overview of the entire data structure (Baehrecke *et al.,* 2004). One output of this type of analysis is the treemap, which was developed to facilitate the simultaneous visualization of hierarchical clustering and the GO framework (Baehrecke *et al.,* 2004). As such, this system of ontological categorization has been commonly employed by numerous *Drosophila* microarray studies in order to simplify data and derive meaning from results (De Gregorio *et al.,* 2001; Johansson *et al.,* 2005; Schlenke *et al.,* 2007; Carpenter *et al.,* 2009; Felix *et al.,* 2012).

Another method that can be utilized to identify useful candidates within the vast quantity of microarray data is network determination; to assess if there are any pair or groups of genes that may interact under particular conditions or constraints. One relatively basic resource that can perform this type of analysis is the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), which attempts to integrate direct experimental evidence of protein-protein/gene-gene interactions, functional grouping of proteins/genes into their respective pathways and predictions of gene-gene/protein-protein interactions (von Mering *et al.,* 2004). A large body of protein-protein associations in STRING are imported from other databases, but the resource also contains a great quantity of predicted interactions that are produced *de novo* via systematic genetic comparisons. As such, sequenced genomes are periodically imported and searched for three genres of genomic context associations: conserved genomic neighbourhoods, gene fusion events and co-occurrence of genes across genomes (von Mering *et al.,* 2004). This combination permits the identification of pairs of genes which appear to be under common selective pressures in a manner which is greater than expected by chance, and which are can therefore assumed to be functionally interacting. Furthermore, a confidence or probability score can be assigned to

each predicted association, which is derived from benchmarking the performance of the predictions against a reference set of validated, true associations; in this case, the functional grouping of proteins maintained at the Kyoto Encyclopedia of Genes and Genomes (KEGG) (von Mering *et al.,* 2004). Any predicted associated for which both genes/proteins are assigned to the same KEGG pathway is considered as a true, positive interaction, meaning that the scores given to associations in STRING represent the chance of finding the interacting candidates within the same KEGG pathway (von Mering *et al.,* 2004) and reflect the probability that the interaction truly exists.

Nevertheless, whilst microarray studies have the potential to identify vast numbers of candidate genes for further validation, it must also be acknowledged that the intrinsic nature of these experiments also generate difficulties when carrying out statistical testing, which must be addressed in order to effectively analyse the resulting data. For instance, a crucial component in the analysis of gene expression microarray experiments is to formulate a list of genes that are differentially expressed upon various biological conditions or treatments, which can then be validated through other experimental techniques to confirm the biological processes involved (Cheng and Pounds, 2007). To determine if genes within these preliminary lists truly display significant changes in expression, statistical hypotheses must be formulated and tested. One problem associated with this process is that a statistical hypothesis must be tested for each gene, and since there are typically thousands of genes within transcriptional profile studies this creates the requirement for massive multiple hypothesis testing (Cheng and Pounds, 2007). Since the probability that false-positive errors may occur increases dramatically with larger numbers of test genes (Reiner *et al.* 2003), as in the case of genome-wide microarray studies, it is necessary to correct the significance values of each result by controlling for the false discovery rate (FDR). In practice, this can be implemented via statistical freeware programs, such as R (Reiner *et al.,* 2002). Therefore, whilst there are numerous tools and resources to simplify the vast quantities of data resulting from microarray

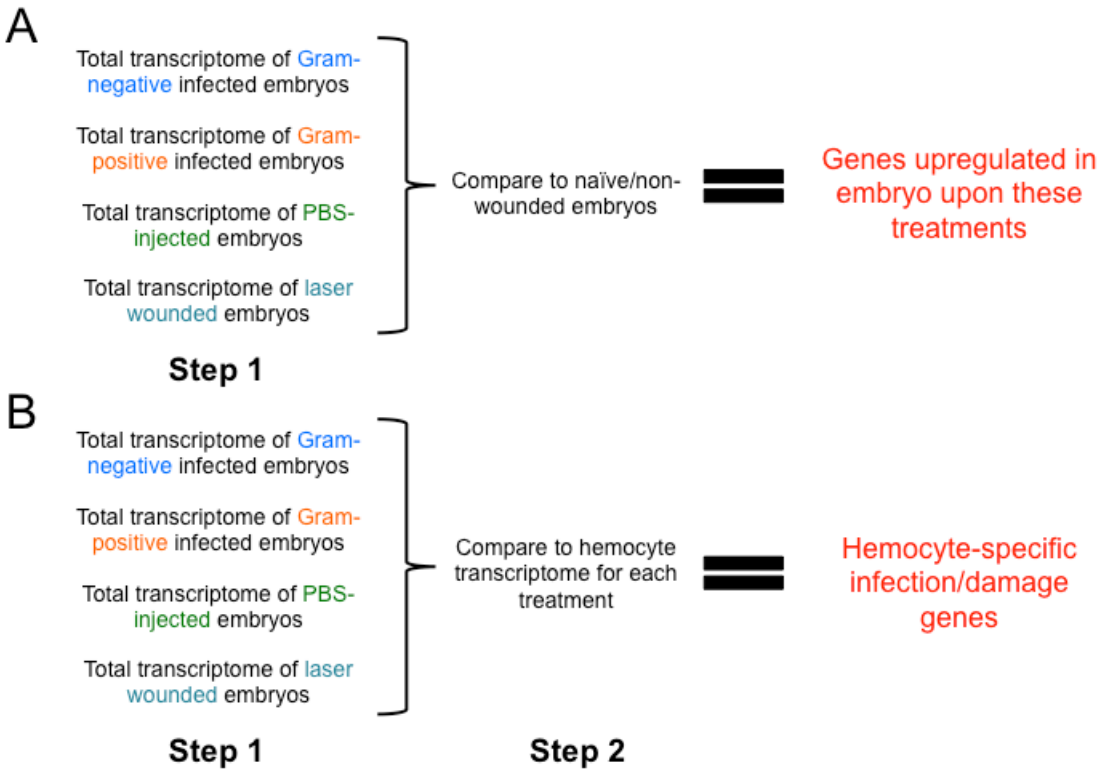
experiments, deriving proper biological relevance requires the correct application of such statistical practices.

4.1.4 Experimental Aims

The final objective of this project was to analyse the content of microarray data obtained as detailed in Chapter 2, to assess the nature of the global transcriptional changes upon bacterial and damage stimuli within the Stage 15 embryo as a whole, as detailed in the strategy depicted in Figure 4.1. By determining which genes were modulated within the embryo upon infection or damage stimuli, induced via microinjection of microbes or sterile laser ablation of embryos respectively, it would be possible to assess the total infected or wounded transcriptomes of the *Drosophila* embryo and to identify potential novel infection- and damage-specific genes. These would ultimately form potential candidate genes for further study. A parallel aim was to more specifically assess the contribution of embryonic hemocytes towards the embryonic damage and immune responses, by examining the transcriptional profile of this immune cell type upon microinjection with sterile endotoxin-free PBS or *Ecc15*. This was achieved by utilizing FACS to isolate hemocytes for RNA processing. By comparison of the hemocyte transcriptome to that of the total embryonic transcriptome, this would permit the identification of hemocyte-specific infection genes. To achieve these objectives, the resulting microarray data was corrected for FDR and subsequently a combination of hierarchical clustering, gene ontology and networking techniques were employed.

Figure 4.1: Schematic of Microarray strategy employed.

A two-phase strategy was employed to study global gene expression within infected and damaged *Drosophila* embryos. In an initial step (**A**), microarray analysis of the total transcriptome of infected and wounded embryos was compared to that of naïve embryos, to determine genes upregulated upon damage or infection. In the second phase, results from Step 1 were compared to the transcriptomes of hemocytes isolated from infected or damaged embryos (**B**). This allowed the identification of hemocyte-specific damage or infection genes.


A
Total transcriptome of Gram-negative infected embryos
Total transcriptome of Gram-positive infected embryos
Total transcriptome of PBS-injected embryos
Total transcriptome of laser wounded embryos
Compare to naïve/non-wounded embryos
Genes upregulated in embryo upon these treatments
Step 1
B
Total transcriptome of Gram-negative infected embryos
Total transcriptome of Gram-positive infected embryos
Total transcriptome of PBS-injected embryos
Total transcriptome of laser wounded embryos
Compare to hemocyte transcriptome for each treatment
Hemocyte-specific infection/damage genes
Step 1
Step 2

4.2. Results

4.2.1 cDNA microarrays are able to detect Immune Gene Modulation within the Stage 15 Drosophila Embryo

The primary aim of these experiments was to more closely examine the global transcriptional responses to damage and infection within the Stage 15 *Drosophila* embryo. To fulfill this aim, cDNA microarray studies were performed to compare the transcriptional profiles of PBS, *Ecc15* (OD=1) or *M. luteus* (OD=1) injected embryos to those of naïve embryos. By comparing the PBS-injected Stage 15 embryo transcriptome to that of the naïve embryo, further knowledge could potentially be gathered regarding the embryonic response to sterile damage. Moreover, comparison between the bacteria injected Stage 15 embryo transcriptomes and the naïve embryos transcriptional profile would permit further study of the complexity of the Stage 15 immune response, as well as the potential identification of any infection specific genes. An initial vsn limma normalization was applied to the subsequent microarray data, and the fold change in gene expression calculated.

Initial analysis sought to determine if changes in expression of immune genes previously characterized in other *Drosophila s*ystems and previous embryonic studies (Chapter 3) could be detected via this methodology (Figures 4.2-4.4). As such, the fold change of AMP gene expression upon PBS, *Ecc15* and *M. luteus* injection was examined (Figure 4.2). Considering the changes in AMP gene expression upon *Ecc15* infection (Figure 4.2A), Gram-negative AMP genes were observed to be predominantly upregulated via microarray analysis. For instance, all *Att* isoforms demonstrated induction upon Ecc15 infection. This was particularly notable for the *AttA* and *AttD* isoforms. Similarly, both *Dpt* isoforms were preferentially induced upon Ecc15 infection, as well as Mtk and the *CecA2* and *CecC* isoforms. Of particular note is the large fold change in Def expression detected, of almost 25-fold. Conversely, *Ecc15* infection did not greatly induce expression of Gram-positive AMPs, such as Drs or its isoforms. By contrast, infection with *M. luteus* appeared to

preferentially induce greater fold changes in *Drs* expression within the Stage 15 embryo, accompanied by decreases in the expression of Gram-negative AMPs, such as *AttA, AttB, CecA2, CecC* and both *Dpt* class members (Figure 4.2B). This, therefore, demonstrates that the specificity of the AMP response observed within the *Drosophila* embryos during previous studies can also be clearly detected via microarray analysis and when applied to individual AMP class genes.

Interestingly, when comparing directly the changes in expression observed with *Ecc15* or *M. luteus* treatment to those observed upon PBS injected, three different patterns emerged, some of which had not previously been detected. When comparing directly levels of AMP gene expression observed with PBS and *Ecc15* treated embryos, in most cases PBS injection stimulated a lower level of AMP gene expression (Figure 4.2A), consistent with previous RT-qPCR results within the Stage 15 embryo (Chapter 3). The only exceptions of this were the downregulated levels of *AttA, AttB, CecA2* and *DptB* recorded upon PBS injection. However, in some cases, as with *Dro*3 and *Dro4, Ecc15* infection induced downregulation and PBS injection initiated an upregulation of AMP gene expression (Figure 4.2A), suggesting a preferential role for these AMPs in a response to damage rather than infection. Of further interest is the pattern detected when comparing fold change of AMP gene expression upon *M. luteus* and PBS treated embryos (Figure 4.2B). In some cases, PBS injection stimulated a higher fold change in AMP gene expression than *M. luteus* infection; as in the case of *Drs.* This would not only infer than these AMP genes can be activated upon damage stimuli, but that *M. luteus* infection reduces AMP gene expression induced via PBS injection.

Having established that modulation of AMP gene expression in the Stage 15 embryo could be effectively detected by the microarray methodology, other families of immune genes were subsequently investigated; to potentially corroborate to the effects observed regarding AMP gene expression. Gene families investigated included the PGRPs, GNBPs (Figure 4.3) and the teps (Figure 4.4). In terms of the expression of individual PGRP family members upon infection with either *Ecc15* (Figure 4.3A) or *M. luteus* (Figure 4.3B),

much variation was noted. For instance, whilst *Ecc15* infection promoted an increase in *PGRP-LC* and *PGRP-LE* expression (Figure 4.3A), *M. luteus* infection resulted in relative downregulation of these PGRP genes (Figure 4.3B). This is consistent with the current paradigm of Drosophila systemic immune signaling, as PGRP-LC and –LE isoforms synergistically function as receptors of the IMD pathway (Takehana *et al.,* 2004; Kaneko *et al.,* 2006). Thus, it is speculative that by downregulating *PGRP-LC* expression, the Stage 15 embryos is able to ensure correct AMP responses according to the appropriate pathogen type. Also of interest is the fact that *PGRP-LF* expression appeared highly induced by *Ecc15* (Figure 4.3A), and *PGRP-SC1* expression by *M. luteus* (Figure 4.3B). Furthermore, examination of the changes in expression of GNBP genes upon *Ecc15* (Figure 4.3A) or *M. luteus* (Figure 4.3B) infection revealed similar patterns. Whilst expression of all GNBP genes was downregulated upon *M. luteus* infection, *Ecc15* induced the upregulation of only *GNBP-3,* which is notable considering its role in Toll pathway activation (Gottar *et al.,* 2006). This may be suggestive of the presence of a feedback mechanism within the Stage 15 embryo that can modulate the expression of individual PGRP genes upon different types of infection. Conversely, the expression of some PGRP genes remained consistent upon infection, regardless of the type of infecting bacterium. For instance, expression of *PGRP-LA, PGRP-LB, PGRP-SA* and PGRP-SC2 was seemingly upregulated upon either *M. luteus* or *Ecc15* infection to similar degrees (Figure 4.3A-B). Similarly, expression of *PGRP-SB2* and *PGRP-SD* was downregulated in a concordant manner by *Ecc15* or *M. luteus* infection (Figure 4.3A-B). This may imply that the function of these genes is somewhat infection aspecific, as concordant modulation of their expression is observed, regardless of microbial genre.

Examination of the expression of PGRP genes upon PBS injection (Figure 4.3A-B) also permitted insight into the modulation of these genes in response to damage stimuli. Of particular interest was the effect of damage on specific PGRP genes, whereby this stimulation induced a change in expression that was distinct from that observed upon infection. For example, whereas *M. luteus* infection induced the expression of PGRP-SC2, PBS injection was

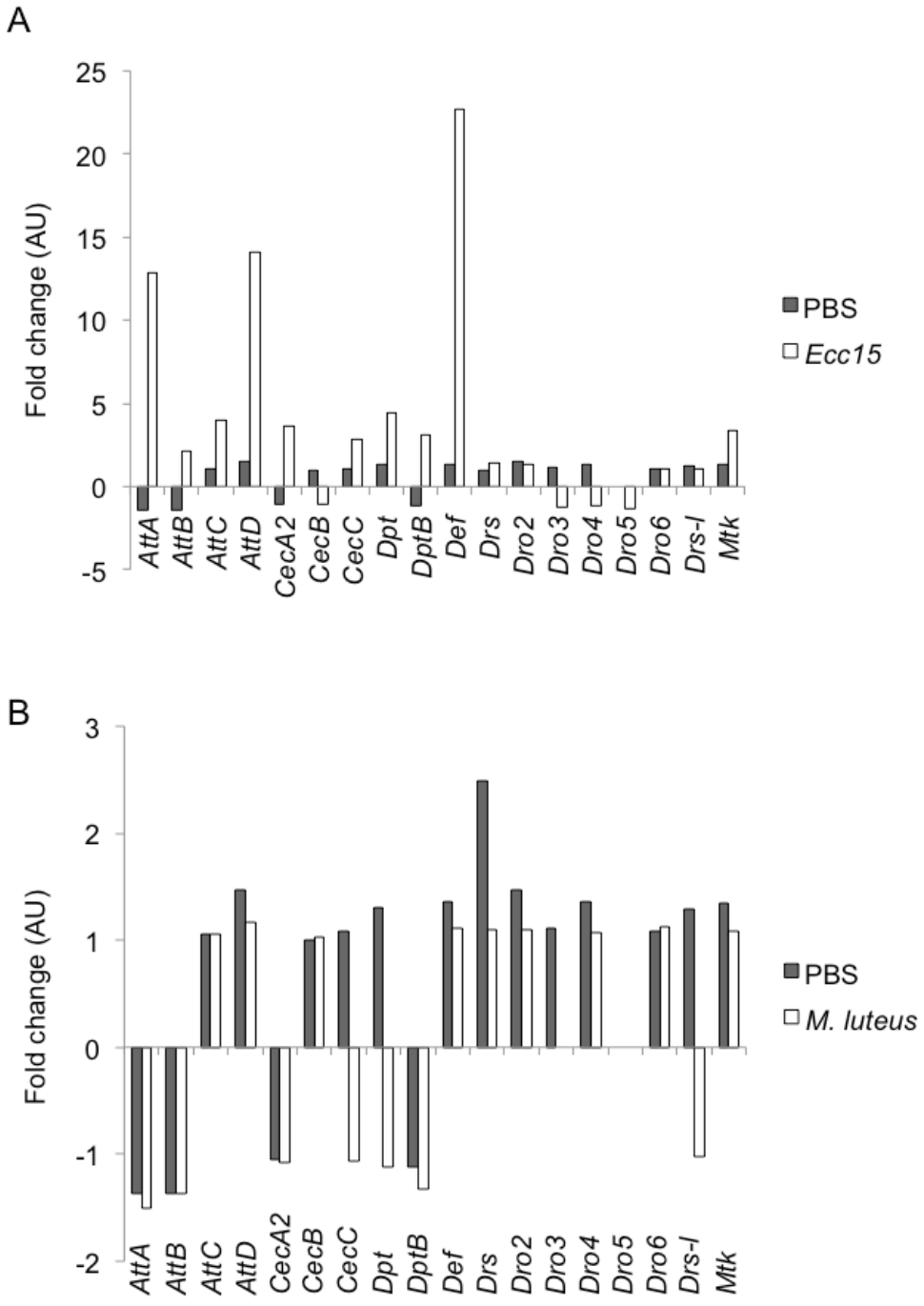
observed to result in downregulation of the PGRP gene, suggesting disparate functions for this family member upon damage and infection in the Stage 15 embryo.

Some of the most interesting results from this preliminary data screening concerns the expression of tep genes upon either *Ecc15* or *M. luteus* infection (Figure 4.4). In some cases, the fold changes in expression observed with *Ecc15* or *M. luteus* infection were relatively concordant. For instance, both *Ecc15* and *M. luteus* induced a decrease in *Mcr* and *TepIV* expression and an increase in *TepIII* expression to similar degrees (Figure 4.4A-B). Conversely, Gram-positive and Gram-negative infections appeared to have a highly differential effect on *TepI* and *TepII* expression. For example, whilst *Ecc15* infection appeared to induce a decrease in *TepI* expression (Figure 4.4A), *M. luteus* infection appeared to increase *TepI* expression (Figure 4.4B). Similarly, *TepII* expression levels were increased by *Ecc15* infection (Figure 3A) and decreased by *M. luteus* infection (Figure 4.4B). Furthermore, it is notable that the changes in *TepI* expression were completely independent of PBS injection, since this treatment alone did not induce any changes in expression (Figure 4.4A-B). This would, therefore, permit the inference that individual tep genes may perform different roles in the response to Gram-negative or Gram-positive pathogens.

Taken together, this data demonstrates that the microarray methodology is able to efficiently detect modulation of immune gene expression within the Stage 15 embryo, with many of the resulting observations being consistent with previous embryonic studies as well as those of other *Drosophila* immunity models. Moreover, the microarray methodology also provides the opportunity to study the individual contribution of immune gene family members to the Stage 15 embryonic immune response; to gain further knowledge concerning the apparently disparate immune responses of the embryo to infection and damage. As such, this preliminary analysis provides the basis for more robust analytical techniques can now be employed to study the genome-wide embryonic transcriptional response to damage and infection stimuli.

Figure 4.2: Modulation in AMP gene expression within the Stage 15 embryo can be detected upon PBS, *Ecc15* or *M. luteus* injection using microarray techniques.

(A-B) Preliminary analysis of microarray data to determine if immune gene modulation could be detected using this methodology, focusing on AMP genes. Data was normalized using vsn limma method, and the fold change between naïve and PBS or bacterial levels was calculated to detect if damage or infection was able to modulate AMP gene expression. The majority of Gram-negative AMP genes were induced upon *Ecc15* infection, as well as *Def* (A); concordant with previous RT-qPCR results. Likewise *M. luteus* infection greatly induced the expression of Gram-positive AMP genes such as those belonging to the *Drs* class (B). In terms of the effect of PBS injection upon the expression of AMP genes, the trends were less defined, with damage downregulating specific AMP gene expression, such as that of the *Att* class (A-B), and upregulating others, including the *Drs* class of AMP genes (A-B). In some cases, the upregulation of AMP genes observed upon PBS expression was higher than that induced via microbial challenge, particularly with *M. luteus* (B), suggesting the infection somehow reduces the AMP response generated by injection damage.


A
Fold change (AU)
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15
10
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-5
AttA
AttB
AttC
AttD
CecA2
CecB
CecC
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DptB
Def
Drs
Dro2
Dro3
Dro4
Dro5
Dro6
Drs-l
Mtk
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AttD
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CecB
CecC
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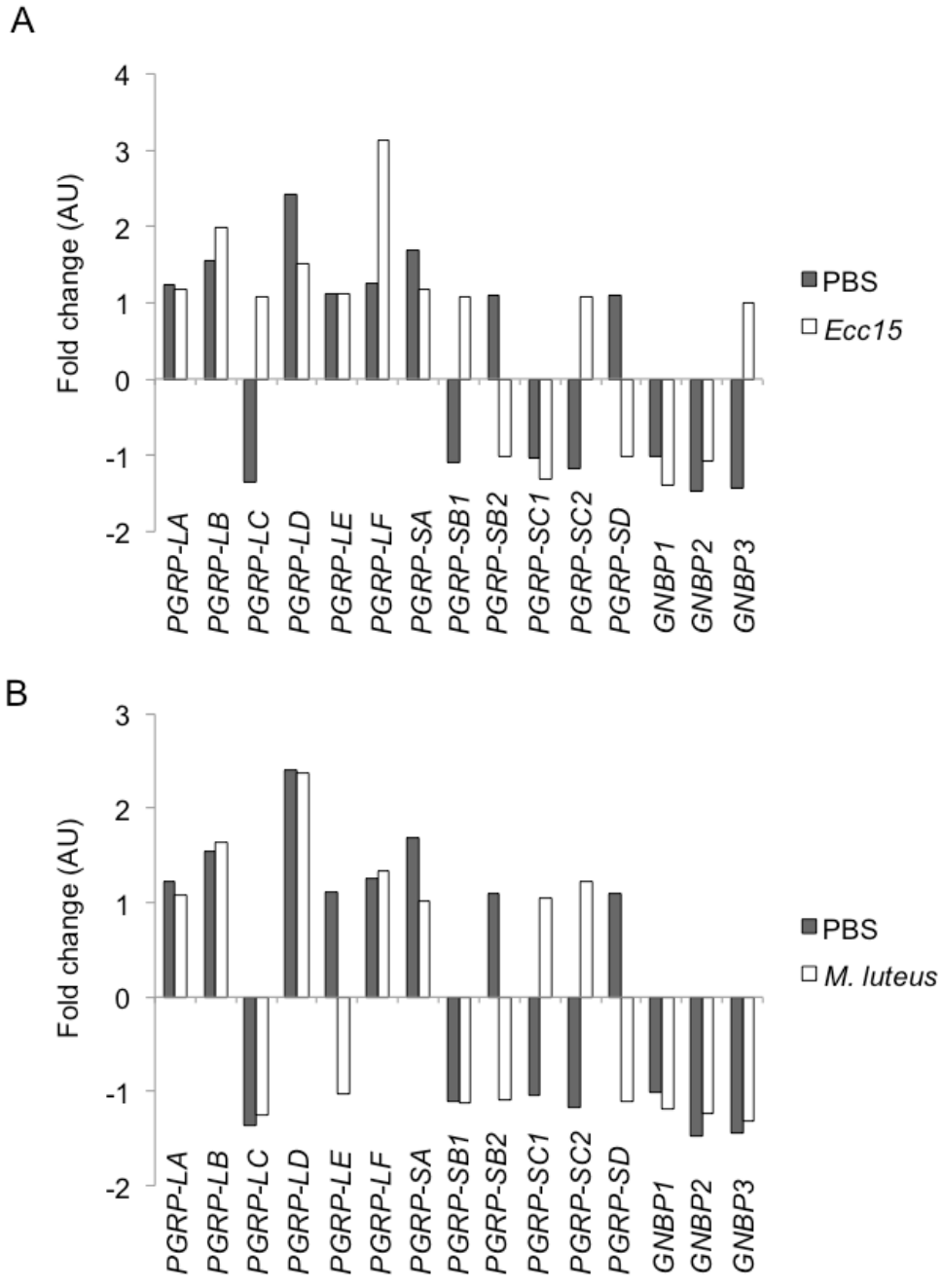
DptB
Def
Drs
Dro2
Dro3
Dro4
Dro5
Dro6
Drs-l
Mtk
PBS
M. luteus

Figure 4.3: PGRP and GNBP gene expression is modulated upon Gram-negative and Gram-positive infection

(A-B) Preliminary analysis of microarray data to determine if PGRP and GNBP gene modulation could be detected using the microarray methodology. Data was normalized using vsn limma method, and the fold change between naïve and PBS or bacterial levels was calculated to detect if damage or infection was able to modulate PGRP and GNBP gene expression. *Ecc15* and *M. luteus* infection produced contrasted effects on the expression of PGRP genes; *Ecc15* infection upregulated PGRP genes involved in IMD signaling, such as *PGRP-LC* and *–LE* (A), whereas *M. luteus* infection downregulated the expression of these isoforms (B). However, the expression of some PGRP family members remained consistent between the two infection types (*PGRP-LA, -LB. LD, -SA, -SB2*; A-B), suggesting a degree of aspecificity within the embryonic immune response. Expression of GNBP genes upon *M. luteus* and *Ecc15* displayed a similar trend, with *GNBP1* and *GNBP2* expression concordantly downregulated by both infection types (A-B), but *GNBP3* expression upregulated by solely *Ecc15* infection (A). PBS injection was also able to induce changes in PGRP gene expression, in some cases distinct from those observed upon bacterial infection (A-B), thus suggesting that individual PGRP genes may be differentially modulated by the embryo upon damage and infection.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-1
-2
PBS
Ecc15
PGRP-LA
PGRP-LB
PGRP-LC
PGRP-LD
PGRP-LE
PGRP-LF
PGRP-SA
PGRP-SB1
PGRP-SB2
PGRP-SC1
PGRP-SC2
PGRP-SD
GNBP1
GNBP2
GNBP3
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Fold change (AU)
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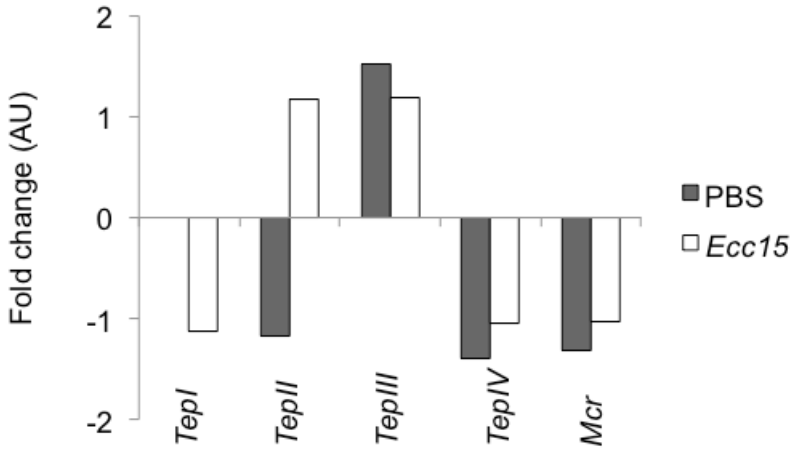
-1
-2
PBS
M. luteus
PGRP-LA
PGRP-LB
PGRP-LC
PGRP-LD
PGRP-LE
PGRP-LF
PGRP-SA
PGRP-SB1
PGRP-SB2
PGRP-SC1
PGRP-SC2
PGRP-SD
GNBP1
GNBP2
GNBP3

Figure 4.4: Expression changes in tep genes upon infection by the Stage 15 embryo can be detected using microarray analysis.

(A-B) Preliminary analysis of microarray data to determine if modulation of tep gene expression could be detected upon PBS, *Ecc15* or *M. luteus* injection using this methodology, focusing on AMP genes. Data was normalized using vsn limma method, and the fold change between naïve and PBS or bacterial levels was calculated. Whilst some tep genes were modulated in a concordant manner by *Ecc15* and *M. luteus* infection (*TepIII, TepIV* and *McR*; A-B), others such as TepI and *TepII* demonstrated differential modulation upon *Ecc15* (A) or *M. luteus* (B) infection, suggestive of different roles for these gene family members upon different types of infection. PBS injection also had differential effects on the expression of tep family members (A-B), implying that damage is able to modulate tep gene expression, and that this modulation is specific to the individual tep gene.

A


2
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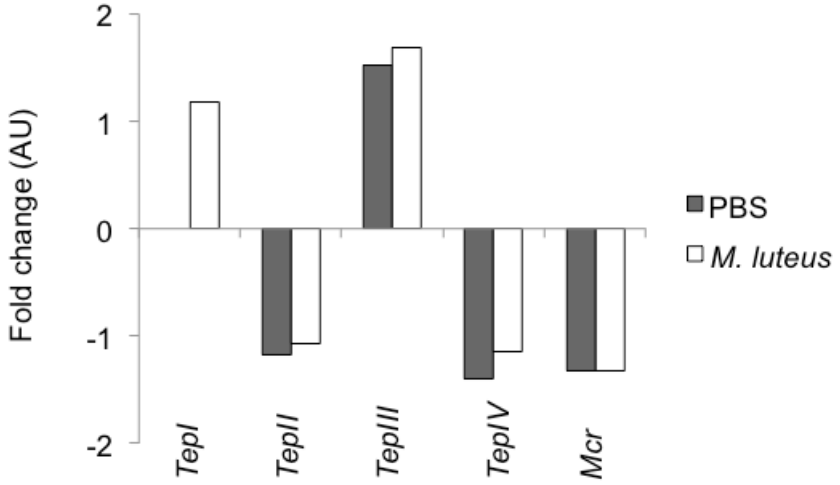
Fold change (AU)
PBS
Ecc15
TepI
TepII
TepIII
TepIV
Mcr

B


2
1
0
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Fold change (AU)
PBS
M. luteus
TepI
TepII
TepIII
TepIV
Mcr

4.2.2 *Ecc15* and *M. luteus* upregulate unique and common subsets of genes within the Stage 15 embryo

In order to effectively differentiate between changes in expression as a result of the injection process and those resulting directly from the bacterial stimulus, comparisons between the expression data collated from PBS injected and bacterial injected Stage 15 embryos were performed. The log fold-change and the FDR corrected significance of observed effects between these data sets was subsequently calculated. Genes whose expression was significantly modulated upon bacterial treatment were initially sub-divided into those experiencing up- or down-regulation. Further categorization permitted the identification of *Ecc15*-unique and *M. luteus*-unique lists of up-regulated genes.

Viewing the list of *Ecc15*-unique upregulated genes (Table A8.1, Appendix 8), there is a clear representation of IMD pathway components. For instance, the AMPs *AttA, AttC* and *AttD* are selectively and significantly upregulated. Furthermore, PGRP-LF, a negative regulator of IMD signaling (Maillet *et al.,* 2008), was also demonstrated to experience upregulation specifically in response to *Ecc15* infection. Other immune pathway involvement in the response mediated specifically to *Ecc15* included the JAK-STAT pathway, inferred by the upregulation of *Glorund* (*Glo*); a gene implicated in the positive regulation of JAK-STAT signaling and wound healing (Muller *et al.,* 2005; Campos *et al.,* 2010). Moreover, the upregulation of *MAP Kinase-Activated Protein Kinase 2* (*MAPK-Ak2*) inferred that signaling via the JNK pathway may also be occurring in response to *Ecc15* infection. Further upregulated *Ecc15*-unique genes included those involved in the melanisation cascade (*pro-phenoloxidase A1, proPO-A1*), phagocytosis (*CG5720*), the encapsulation of foreign targets (*Alpha-mannosidase, Alpha-man-I; UDP-GlcNAc:a-3-D-mannoside-β-1,2-N-acetylglucosaminyltransferase I, Mgat1; glial cells missing, gcm2*) or autophagy (*Autophagy specific gene 9, Atg9; Bruce*). Interestingly, *Bruce* has been demonstrated to be a negative regulator of autophagy (Hou *et al.,* 2008; Nezis *et al.,* 2010) hence its upregulation would suggest an inhibition of the autophagic process, which is not necessarily concordant with the

requirements of the embryonic host. The *Ecc15*-specific upregulation of *elevated during infection* (*edin*) within the embryo was also notable, since this gene has been characterized to be upregulated upon infection but as of yet has had no particular function attributed (Vanha-aho *et al.,* 2012). Other genes shown to be up-regulated in a *Ecc15*-specific manner did not have any particular immune connotations and their functions appeared relatively disparate. These included *Hermansky-Pudlak syndrome 4 ortholog* (*HPS4*), which is involved in the negative regulation of gene silencing (Lee *et al.,* 2009), *Vacuolar protein sorting 26* (*Vps26*), which is involved in lysosome organization and vacuolar transport (Saftig *et al.,* 2009), and *Rab9*, which is involved in late endosomal vesicular transport (Zhang *et al.,* 2007). Furthermore, it is also noteworthy that genes involved in neurotransmitter secretion (*Rab3 interacting molecule, Rim*), dendrite guidance (*Sema-2a*), axonal mitochondrial distribution (*milton, milt*) and neuron development (*sno oncogene, snoo*) were up-regulated by *Ecc15* infection, reflecting a potential relationship between *Ecc15* infection and nervous system development and activity. However, 24% of the genes identified as being uniquely upregulated upon *Ecc15* infection are currently of unknown function (Table A8.1, highlighted in green), and thus it is not possible to comment on their relevance to the embryonic *Ecc15* transcriptional response.

Similar broad conclusions can be drawn regarding the genes specifically up-regulated by *M. luteus* infection (Table A8.2, Appendix 8). *M. luteus* infection of Stage 15 embryos resulted in the specific upregulation of immune genes, such as *Edysone-inducible gene 71Eh* (*Eig71Eh*); a gene whose precise function remains elusive, but encodes a protein which is similar to mammalian defensins (Zraly and Dingwall, 2012), thus implying an immune function. *TepI* expression was also demonstrated to be up-regulated specifically as a result of *M. luteus* infection, suggesting a potential specificity in the opsonisation of *M. luteus* upon infection and hinting at an upregulation of JAK-STAT signaling upon this type of infection (Lagueux *et al.,* 2000). Moreover, the upregulation of *CG34127* and *CG6652* may suggest a specific impact of *M. luteus* on phagocytosis, since these genes have previously been associated with this this process upon *C. albicans* infection (Stroschein-Stevenson *et al.,* 2006)

However, the *M. luteus*-specific upregulation of genes associated with non-immune functions would suggest that this type of infection also impacts other biological systems within the Stage 15 embryo. For instance, up-regulation of the *Activating Transcription Factor 2* (*Atf-2*) and *ribosomal protein L8* (*RpL8*) would also indicate an increase in MAPK signaling and insulin signaling respectively. The gene hits associated with *M. luteus* infection also displayed an interesting connection to nervous system development, such as neurogenesis (*threonyl-tRNA synthetase, Aats-thr*) and neuron fate commitment (*brain specific homeobox, bsh*), but also to spermatogenesis (*Male-specific transcript 36Fa, Mst36Fa*) and sperm competition (*Accessory gland protein 53Ea, Acp53Ea*). Up-regulated expression of *mastermind* (*mam*) may also indicate an impact of *M. luteus* on stem cell differentiation, and upregulation of olfactory binding proteins and receptors (*Obp57e, Or85d, Or98a*) may suggest an impact on the sensing of chemical stimuli, such as pheromone. It is also of interest that *M. luteus* specifically upregulated two distinct *Cytochrome P450 oxygenase* (*Cyp)* genes (*Cyp308a1 and Cyp309a1*), which play roles in the oxidation-reduction process and also display heme binding properties (Flybase Curators *et al.,* 2004), as well as a glucose transporter (*sut3*). Similar to the nature of candidates upregulated specifically by *Ecc15* infection, many genes uniquely upregulated by *M. luteus* (27%) are also still awaiting assignment of both a precise molecular and more broad biological function.

To determine the potential overlap of genes up-regulated by both types of infection, comparison of the top 200 most significantly up-regulated genes upon either *Ecc15* or *M. luteus* infection was performed (Figure 4.5). Of these genes, 73 (36.5%) were commonly up-regulated by both *Ecc15* and M*. luteus* infection (Figure 4.5A). A list of these common genes can be viewed in Figure 4.5B. The majority of these genes did not appear to be specific to any immune function. For instance, genes involved in regulation of transcription and mRNA transport, such as *ENL/AF9-related* (*ear*), *paired* (*prd*) and *small bristles* (*sbr*), were commonly up-regulated. The commonly up-regulated genes also had functions pertaining to ion transport (*Eag-like K^+ channel, elk; N,K-ATPase Interacting, NKAIN*), cellular membrane organization (*endophilin B, endoB;*

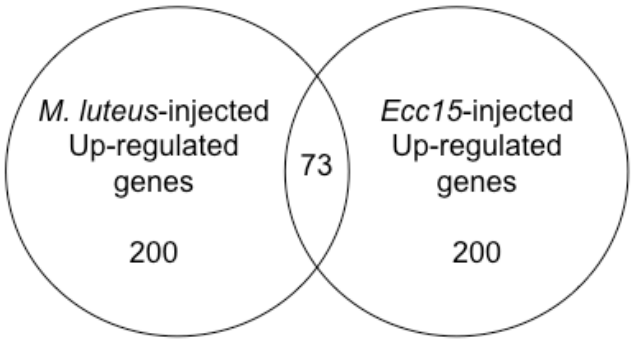
four way stop, fws), DNA repair (*Snm1; (6-4)photolyase, phr6-4*), the response to stress (*locomotion defects, loco*), regulation of the cell cycle (*Invadolysin*) and protein phosphorylation (*Protein Kinase-like 17E*). The only commonly up-regulated gene with annotations related to immune functionality was *spatzle 4* (*spz4*), a ligand for Toll pathway activation. Interestingly, genes implicated in the determination of adult lifespan were also commonly up-regulated by *M. luteus* and *Ecc15* infection, including *snazarus* (*snz*) and the *SIFamide receptor* (*NIFR)*, hinting at a possible impact of these infections upon *Drosophil*a aging. Moreover, genes with roles in the development of specific tissues were also commonly up-regulated by *Ecc15* and *M. luteus* including *capricious* (*caps*), a gene implicated in the development of the tracheal system (Krause *et al.,* 2006) and motor axon guidance (Kurusu *et al.,* 2008), and *nimA-like kinase* (*niki*), which is involved in neuron projection morphogenesis (Sepp *et al.,* 2008).

Therefore, these results would suggest that, as well as specifically upregulating genes with established immune roles, *Ecc15* and *M. luteus* infections also appears to induce the expression of genes with more disparate functions; highlighting the widespread impact that this infection type has on the Stage 15 embryonic transcriptome. Furthermore, it provides an effective demonstration that subsets of genes are upregulated specifically in response to Gram-positive or Gram-negative infection, or in an aspecific manner, in the Stage 15 embryo.

Figure 4.5: Comparison of Genes Modulated upon *Ecc15* and *M. luteus* infection reveals a common subset of Upregulated Genes

(A-B) A common subset of genes is up-regulated by *Ecc15* and *M. luteus* infection within the Stage 15 *Drosophila* embryo. Of the 200 most significantly upregulated genes upon either *Ecc15* or *M. luteus* infection, 73 (36.5%) are common to both infection types (A). These commonly upregulated genes include regulation of transcription, ion transport, cellular membrane organization, DNA repair, the response to stress, regulation of the cell cycle, protein phosphorylation, adult lifespan, nervous and tracheal system development (B). However 22% of genes implicated in this comparison currently have no annotations concerning their molecular function or the biological process that they may be involved in (B, highlighted in green).

A



B

	GRAM-POSITIVE *M. luteus*		GRAM-NEGATIVE *Ecc15*	
Gene	**Log Fold-change (AU)**	**P value**	**Log Fold-Change (AU)**	**P value**
AdoR	2.130	0.004	1.154	0.011
bun	1.674	0.014	2.060	0.002
caps	1.232	0.021	1.210	0.008
CG10252	2.418	0.004	2.126	0.001
CG10321	1.585	0.022	1.627	0.010
CG10737	1.199	0.017	1.274	0.012
CG12099	1.405	0.020	1.708	0.005
CG1233	1.334	0.008	1.533	0.004
CG12950	1.356	0.009	1.692	0.005
CG13506	1.291	0.018	1.462	0.012
CG13843	1.642	0.016	2.670	0.006
CG16771	1.262	0.009	1.279	0.010
CG17450	3.941	0.015	2.004	0.015
CG17838	2.803	0.002	2.474	0.003
CG2224	1.477	0.015	1.500	0.014
CG2765	1.352	0.007	1.385	0.010
CG2943	1.117	0.021	1.078	0.017
CG31205	3.875	0.010	2.994	0.009
CG3194	1.573	0.012	1.694	0.009
CG32549	1.783	0.018	1.511	0.016
CG3309	1.935	0.013	2.042	0.015
CG33233	3.415	0.007	2.443	0.011
CG3419	1.375	0.017	1.507	0.007
CG34351	1.904	0.002	1.744	0.009
CG34383	1.423	0.022	1.529	0.018
CG42327	1.642	0.005	1.721	0.003
CG42575	1.592	0.012	1.608	0.010
CG42594	3.182	0.020	2.873	0.010
CG42750	3.324	0.014	3.583	0.004
CG43155	2.414	0.009	1.729	0.013
CG43695	5.879	0.009	4.970	0.000
CG7139	1.127	0.015	1.189	0.009
CG7510	1.104	0.022	1.159	0.018
CG7611	1.661	0.022	1.891	0.009
CG8516	1.760	0.010	1.669	0.018
CG8578	1.703	0.010	1.708	0.009
CG8671	1.372	0.014	1.474	0.005
CG8757	3.962	0.003	3.559	0.000
CG8801	1.414	0.016	1.622	0.008

CG9121	1.355	0.008	1.597	0.003
CG9304	1.239	0.012	1.285	0.009
Dgk	1.410	0.011	1.480	0.009
Dic3	2.792	0.005	4.573	0.006
Eaf	1.943	0.016	2.234	0.007
ear	1.996	0.010	2.322	0.008
elk	2.353	0.013	2.389	0.002
endoB	1.254	0.016	1.326	0.010
fws	1.541	0.013	1.578	0.011
inaF-BinaF-D	1.397	0.016	1.448	0.012
Invadolysin	1.502	0.008	1.528	0.010
Klp68D	1.498	0.006	1.492	0.008
KP78bKP78a	1.693	0.007	2.321	0.012
loco	1.610	0.006	1.684	0.002
Lsp2	3.210	0.003	3.279	0.000
niki	7.561	0.000	7.108	0.000
NKAIN	1.477	0.017	1.475	0.014
Nmdar2	1.291	0.019	1.617	0.009
osk	2.525	0.008	1.532	0.008
Patj	2.151	0.005	2.114	0.006
phr6-4	2.798	0.006	2.466	0.007
Pi3K68D	0.979	0.022	1.028	0.015
pigeon	1.714	0.006	1.845	0.003
Pk17E	1.245	0.014	1.084	0.017
prd	5.516	0.008	6.255	0.001
pug	1.273	0.012	1.356	0.016
Rop	1.560	0.011	1.605	0.012
sbr	1.440	0.020	1.577	0.017
SIFR	3.854	0.000	3.092	0.001
Snm1	1.724	0.006	1.396	0.011
snz	1.704	0.006	1.743	0.005
SP2637	1.614	0.013	1.726	0.007
spz4	1.704	0.008	1.562	0.010
stmA	1.393	0.007	1.433	0.012

Names highlighted in green denote genes of unknown function.

4.2.3. Gene ontology analysis of *Ecc15*-modulated genes reveals an enrichment of genes with roles in neuronal signaling, DNA replication and protein trafficking within the Stage 15 embryo

To gain further knowledge concerning the potential function of the genes both up- and down-regulated upon *Ecc15* infection, and hence of the global transcriptional response of the Stage 15 embryo to Gram-negative infection, GO analysis was performed on the lists of genes calculated to be significantly up- or down-regulated by *Ecc15* infection, compared to those modulated by PBS infection, after correction for FDR. The Gene Ontology Enrichment Analysis and Visualisation Tool (GOrilla) was employed to search for enriched GO terms relating to the biological processes associated with the lists of genes. The identified GO terms were then sorted based on the significance of GO term enrichment below a threshold of $p \leq 0.05$.

Whilst large numbers of genes upregulated by *Ecc15* infection were associated with more general GO biological process terms, such as biological regulation and single organism transport (Table 4.1), greater insight into the potential enrichment of biological processes was attained by observing more specific terms. Relatively large numbers of genes were associated with transport terms, such as synaptic vesicle function, exocytosis and axon cargo transport, suggesting roles in neurotransmitter release or protein trafficking. A large number of genes were also attributed with a more GPCR signaling function, which could also be widely suggestive of some manner of neuronal function. Conversely, only one general GO biological process term was enriched within the genes significantly downregulated upon *Ecc15* infection (Table 4.2); this pertained to the sensation of chemical stimuli, from which is was difficult to assign any more precise function.

To attempt to further derive information concerning the collective functions of up- and down-regulated genes upon *Ecc15* treatment within the Stage 15 embryo, STRING 9.0 was used to determine if any GO terms relating to molecular function and the cellular component concerned could be assigned to these genes. Numbers of genes associated with the each distinct GO term

were counted and enrichment P-values where p≤0.05 were corrected for FDR, generating a q-value. Only those terms with a value of q<1.00 were accepted. Considering the molecular function GO terms associated with the genes whose expression was significantly up-regulated upon *Ecc15* infection (Table 4.3), the list of potential terms generated is extensive; there are terms covering a range of molecular functions, suggesting that the precise functions of *Ecc15*-upregulated genes are relatively diverse and that the global embryonic response to this infection is highly complex. However, broadly speaking, these terms could be sub-divided into 4 major functional areas: DNA or RNA binding, channel and transporter activity, enzymatic functions and cytoskeletal regulation, labeled in red, green, blue and purple in Table 4.3 respectively. In particular, the numbers of genes associated with DNA binding GO terms was high; for instance, 87 genes were associated with the more general nucleotide binding function, with 65 being more specifically associated with purine nucleotide binding. Also of note was the high number of up-regulated genes associated with the catalytic activity term (209); scanning Table 4.3, it is clear that the associated enzymatic activity is greatly diverse, including oxidoreductase, phosphotransferase, monophenol monoxygenase, transferase and endopeptidase activity terms. Of further interest was the large number of up-regulated genes that demonstrated association with channel and transporter actvitiy; 16 of these terms are associated with ion transport, either via voltage-gated channels or more generically assigned transmembrane transporters. To an extent, the GO cellular component terms assigned to *Ecc15* up-regulated genes agreed with these proposed molecular function (Table 4.5). Whilst *Ecc15*-upregulated genes appeared to be associated with a rather extensive list of cellular components, many were assigned with terms relating to the nervous system, such as the synapse, synaptic vesicle, axon terminus, neuron projection and synaptic membrane. This potentially agreed with the high numbers of genes displaying association with ion channel and transporter molecular functions (Table 4.3). Many genes were also attributed with terms relating to protein synthesis and export, such as the clarithrin coated vesicle, endosome, Golgi apparatus and multivesicular body, which may also potentially explain the large numbers of genes associated with enzymatic GO molecular terms. Furthermore, genes were associated with the

nuclear origin of replication recognition complex and the Arp2/3 protein complex GO cellular component terms, in concordance with the assignment of GO molecular function terms pertaining to DNA binding and cytoskeletal protein binding.

Moreover, genes significantly downregulated upon *Ecc15* infection also demonstrated a high association with transport-related GO molecular functions (Table 4.4), with many genes attributed GO terms relating to channel, voltage-gated ion channel and substrate-specific channel activity. Other genes were assigned to terms relating to peptidase or hydrolase activity, suggesting enrichment of genes involved in proteolysis. 5 genes were also associated with a DNA helicase function, suggesting interaction with DNA in some capacity. However, it is notable that the range of molecular functions associated with downregulated genes was much smaller than that observed with *Ecc15*-upregulated genes, suggesting a much more concentrated response. This is also reflected in the relatively limited number of GO cellular component terms associated with genes downregulated upon *Ecc15* infection (Table 4.6), in contrast to the apparently more extensive distribution of *Ecc15* upregulated genes (Table 4.5). Moreover, *Ecc15* downregulated genes further functionally contrasted with *Ecc15* upregulated genes in terms of the nature of the GO cellular terms subsequently assigned to them. Whilst upregulated genes were assigned terms associated with neural components, downregulated genes were associated with terms relating to extracellular space and the chorion (Table 4.6). Moreover, compared to the enrichment of upregulated genes within the origin of replication, downregulated genes were associated with the GINS complex, a component of the replisome required for progression (Kamada, 2012) and within the pre-initiation complex. Hence, whilst both *Ecc15* upregulated and downregulated genes demonstrated similar transporter, channel or DNA interacting functions, their principle localisations appeared to be relatively different, suggestive of different functions. Taken together, these results suggest that genes upregulated by *Ecc15* within the Stage 15 embryo are enriched for neuronal, DNA replication and protein synthesis and trafficking functions. In contrast, the functionality of *Ecc15*-downregulated genes may be much more restricted, with some genes

potentially playing a role within the replisome, although the precise nature of these global functions remains relatively unclear.

Table 4.1: Biological process GO terms associated with genes upregulated upon *Ecc15* infection

GO Biological Process Term	Number of Genes (AU)	P-value	q-value
Synaptic vesicle transport	12	3.33E-06	1.75E-02
Single-organism transport	71	1.66E-05	4.26E-02
Biological regulation	152	1.99E-04	3.47E-01
Exocytosis	7	2.16E-04	2.84E-01
Transport	75	3.00E-04	3.15E-01
Axon cargo transport	5	6.42E-04	5.62E-01
GPCR signaling pathway	17	9.63E-04	7.22E-01

Table 4.2: Biological process GO terms associated with genes downregulated upon *Ecc15* infection

GO Biological Process Term	Number of Genes (AU)	P-value	q-value
Detection of chemical stimulus	11	6.68E-05	3.43E-01

Table 4.3: Molecular function GO terms associated with genes upregulated upon *Ecc15* infection

GO Molecular Function Term	Number of Genes (AU)	P-value	q-value
Purine ribonucleoside triphosphate binding	66	1.29E-05	9.30E-03
Nucleotide binding	87	1.77E-05	9.30E-03
Purine ribonucleotide binding	65	2.37E-05	9.30E-03
Ribonucleotide binding	65	2.37E-05	9.30E-03
Purine nucleotide binding	65	2.75E-05	9.30E-03
Small molecule binding	90	7.01E-05	2.07E-02
ATPase activity, coupled to transmembrane movement of substances	17	1.72E-04	4.07E-02
Hydrolase activity, catalyzing transmembrane movement of substances	17	1.72E-04	4.07E-02
ATP binding	52	2.61E-04	5.28E-02
Adenyl ribonucleotide binding	52	2.80E-04	5.28E-02
Adenyl nucleotide binding	52	2.90E-04	5.28E-02
ATPase activity, coupled to movement of substances	16	3.45E-04	5.82E-02
Transmembrane transporter activity	53	5.39E-04	8.49E-02
Cation channel activity	14	6.73E-04	9.94E-02
Voltage-gated cation channel activity	7	8.61E-04	1.20E-01
Cation transmembrane transporter activity	32	9.58E-04	1.23E-01
P-P-bond-hydrolysis-driven transmembrane transporter activity	17	1.11E-03	1.23E-01
ATPase activity, coupled to transmembrane movement of ions	11	1.22E-03	1.25E-01
Potassium channel activity	6	1.33E-03	1.31E-01
Inorganic cation transmembrane transporter activity	26	1.72E-03	1.36E-01
Transporter activity	56	1.80E-03	1.69E-01
Cation-transporting ATPase activity	6	1.97E-03	1.69E-01
Substrate-specific transmembrane transporter activity	44	1.98E-03	1.69E-01

Catalytic activity	209	2.00E-03	1.69E-01
SAM domain binding	2	2.20E-03	1.69E-01
Cyclic nucleotide-dependent guanyl-nucleotide exchange factor activity	2	2.20E-03	1.74E-01
O-acyltransferase activity	5	2.44E-03	1.74E-01
Ion transmembrane transporter activity	37	2.45E-03	1.81E-01
Ion channel activity	18	2.69E-03	1.81E-01
ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	10	2.81E-03	1.93E-01
Voltage-gated potassium channel activity	5	3.04E-03	1.95E-01
Kinase activity	29	3.04E-03	2.00E-01
Nucleoside-triphosphatase activity	40	3.41E-03	2.00E-01
Substrate-specific channel activity	18	3.59E-03	2.18E-01
Metal ion transmembrane transporter activity	19	3.79E-03	2.23E-01
Voltage-gated ion channel activity	7	4.09E-03	2.29E-01
Phosphotransferase activity, alcohol group as acceptor	27	4.14E-03	2.33E-01
Transferase activity, transferring phosphorus-containing groups	37	4.64E-03	2.33E-01
Pyrophosphatase activity	40	4.66E-03	2.43E-01
Protein binding	82	4.80E-03	2.43E-01
Kinesin binding	3	4.84E-03	2.43E-01
Non-membrane spanning protein tyrosine kinase activity	3	4.84E-03	2.43E-01
Substrate-specific transporter activity	45	5.11E-03	2.43E-01
Channel activity	18	5.25E-03	2.48E-01
Hydrolase activity, in phosphorus-containing anhydrides	40	5.75E-03	2.48E-01
Monophenol monooxygenase activity	2	6.41E-03	2.66E-01
Protein histidine kinase activity	2	6.41E-03	2.70E-01
Oxidoreductase activity	2	6.41E-03	2.70E-01
Two-component sensor activity	2	6.41E-03	2.70E-01
Receptor signaling protein	7	6.70E-03	2.70E-01

activity			
Cytoskeletal protein binding	19	9.19E-03	2.78E-01
Protein kinase activity	21	9.59E-03	3.75E-01
Transferase activity	65	1.03E-02	3.84E-01
Transferase activity, transferring acyl groups other than amino-acyl groups	13	1.22E-02	4.06E-01
Transmembrane receptor protein serine/threonine kinase activity	2	1.24E-02	4.58E-01
Transforming growth factor beta-activated receptor activity	2	1.24E-02	4.58E-01
Oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor	2	1.24E-02	4.58E-01
Protein tyrosine phosphatase activity	6	1.30E-02	4.58E-01
Heat shock protein binding	6	1.62E-02	4.72E-01
Adenylyltransferase activity	3	1.65E-02	5.79E-01
Ras guanyl-nucleotide exchange factor activity	5	1.79E-02	5.83E-01
Guanyl-nucleotide exchange factor activity	7	1.83E-02	6.23E-01
GTP binding	14	1.93E-02	6.28E-01
Divalent inorganic cation transmembrane transporter activity	6	1.98E-02	6.46E-01
Calcium-transporting ATPase activity	2	2.01E-02	6.46E-01
Calcium-dependent cysteine-type endopeptidase activity	2	2.01E-02	6.46E-01
Structural constituent of cytoskeleton	5	2.02E-02	6.46E-01
Proton-transporting ATPase activity, rotational mechanism	4	2.10E-02	6.46E-01
Nucleotidyltransferase activity	8	2.40E-02	6.60E-01
Single-stranded RNA binding	4	2.42E-02	7.44E-01
Actin binding	11	2.54E-02	7.44E-01
GTPase activity	12	2.62E-02	7.70E-01
Potassium ion transmembrane transporter activity	6	2.63E-02	7.78E-01

Polynucleotide adenylyltransferase activity	2	2.92E-02	7.78E-01
1-acylglycerol-3-phosphate O-acyltransferase activity	2	2.92E-02	8.01E-01
Transmembrane receptor protein tyrosine phosphatase activity	2	2.92E-02	8.01E-01
Phosphotransferase activity, nitrogenous group as acceptor	2	2.92E-02	8.01E-01
Carnitine O-acyltransferase activity	2	2.92E-02	8.01E-01
Hydrogen-exporting ATPase activity, phosphorylative mechanism	6	3.41E-02	8.01E-01
Calcium ion transmembrane transporter activity	5	3.46E-02	9.27E-01
Guanyl ribonucleotide binding	13	3.50E-02	9.29E-01
Guanyl nucleotide binding	13	3.66E-02	9.29E-01
Transferase activity, transferring acyl groups	13	3.66E-02	9.43E-01
ATPase activity	23	3.67E-02	9.43E-01
Alkali metal ion binding	2	3.96E-02	9.43E-01
Potassium ion binding	2	3.96E-02	9.84E-01
Pyruvate kinase activity	2	3.96E-02	9.84E-01

Functions highlighted in red, green, blue and purple represent GO terms broadly pertaining to DNA or RNA binding, channel and transporter activity, enzymatic functions and cytoskeletal regulation respectively.

Table 4.4: Molecular function GO terms associated with genes downregulated upon *Ecc15* infection

GO Molecular Function Term	Number of genes (AU)	P-value	q-value
Hydrolase activity	71	1.14E-03	7.26E-01
Channel activity	14	1.32E-03	7.26E-01
Serine-type peptidase activity	18	1.60E-03	7.26E-01
Serine hydrolase activity	18	1.73E-03	7.26E-01
Serine-type endopeptidase activity	17	1.86E-03	7.26E-01
Ion channel activity	13	2.15E-03	7.26E-01
Substrate-specific channel activity	13	2.72E-03	7.91E-01
DNA helicase activity	5	3.53E-03	7.91E-01
Ligand-gated ion channel activity	8	3.73E-03	7.91E-01
Peptidase activity, acting on L-amino acid peptides	27	4.02E-03	7.91E-01

Table 4.5: Cellular component GO terms associated with genes upregulated upon *Ecc15* infection

GO Cellular Component Term	Number of Genes (AU)	P-value	q-value
Synapse	19	1.36E-04	6.90E-02
Axon terminus	7	2.74E-04	6.90E-02
Neuron projection terminus	7	2.74E-04	6.90E-02
Cell projection	20	3.52E-04	6.90E-02
Axon	11	4.00E-04	6.90E-02
Terminal button	6	5.93E-04	7.70E-02
Synapse part	15	6.46E-04	7.70E-02
Neuron projection	13	7.70E-04	7.70E-02
Axon part	7	8.04E-04	7.70E-02
Female germline ring canal outer rim	2	2.30E-03	1.63E-01
Endosome	8	2.40E-03	1.63E-01
Voltage-gated potassium channel complex	4	2.65E-03	1.63E-01
Membrane-bounded vesicle	13	2.65E-03	1.63E-01
Clathrin-coated vesicle	9	3.63E-03	2.09E-01
Cation channel complex	5	4.08E-03	2.20E-01
Coated vesicle	10	4.72E-03	2.39E-01
Cytoplasm	130	5.24E-03	2.51E-01
Golgi apparatus	14	6.75E-03	3.06E-01
Presynaptic membrane	3	7.43E-03	3.09E-01
Synaptic vesicle	8	7.53E-03	3.09E-01
Vesicle	13	1.06E-02	4.16E-01
Ion channel complex	6	1.13E-02	4.24E-01
Golgi apparatus part	9	1.20E-02	4.31E-01
Cytoplasmic membrane-bounded vesicle	11	1.34E-02	4.61E-01
Proton-transporting two-sector ATPase complex	7	1.55E-02	5.13E-01
Early endosome	4	1.64E-02	5.16E-01
Endomembrane system	17	1.71E-02	5.16E-01
Membrane fraction	3	1.75E-02	5.16E-01
Protein complex	94	1.89E-02	5.16E-01
Cell	248	1.92E-02	5.16E-01
Cell part	248	1.92E-02	5.16E-01
Glucosidase II complex	2	2.09E-02	5.45E-01
Cytoplasmic vesicle	11	2.74E-02	6.95E-01
Female germline ring	2	3.03E-02	7.13E-01

canal			
Arp2/3 protein complex	2	3.03E-02	7.13E-01
Synaptic membrane	5	3.06E-02	7.13E-01
Golgi membrane	5	3.39E-02	7.69E-01
Vacuole	7	3.54E-02	7.82E-01
Cell projection part	8	3.74E-02	8.06E-01
Multivesicular body	2	4.11E-02	8.06E-01
Nuclear origin of replication recognition complex	2	4.11E-02	8.06E-01
Origin recognition complex	2	4.11E-02	8.06E-01
M band	2	4.11E-02	8.06E-01
Proton-transporting two-sector ATPase complex, catalytic domain	4	4.31E-02	8.25E-01
ATP-binding cassette (ABC) transporter complex	5	4.92E-02	9.04E-01

Table 4.6: Cellular component GO terms associated with genes downregulated upon *Ecc15* infection

GO Cellular Component Term	Number of Genes (AU)	P-value	q-value
GINS complex	3	6.52E-05	2.81E-02
DNA replication Preinitiation complex	3	6.52E-05	2.81E-02
Dynein complex	6	5.62E-04	1.50E-01
Extracellular space	18	6.96E-04	1.50E-01
Extracellular region	32	9.29E-04	1.60E-01
External encapsulating structure	6	2.22E-03	3.19E-01
Chorion	5	2.66E-03	3.28E-01
Extracellular region part	19	4.57E-03	4.93E-01

4.2.4. Gene ontology analysis of *M. luteus*-modulated genes reveals an enrichment of genes with roles in defense, ion transport and neurotransmission within the Stage 15 embryo

To further assess the potential functions of the genes both up- and down-regulated upon *M. luteus* infection, and hence of the global transcriptional response of the Stage 15 embryo to Gram-positive infection, GO analysis was performed on the lists of genes calculated to be significantly up- or down-regulated by *M. luteus* infection, compared to those modulated by PBS infection, after correction for FDR. The Gene Ontology Enrichment Analysis and Visualisation Tool (GOrilla) was employed to search for enriched GO terms relating to the biological processes associated with the lists of genes. The identified GO terms were then sorted based on the significance of GO term enrichment below a threshold of $p \leq 0.05$.

In contrast to the more limited biological process GO terms that were enriched upon *Ecc15* infection of Stage 15 embryo, those enriched by genes both up and down-regulated upon *M. luteus* infection demonstrated a greater diversity in terms of biological function; with 29 different GO terms associated with *M. luteus* upregulated genes as opposed to the 7 GO terms enriched by *Ecc15* upregulated genes. However, considering the biological process GO terms attributed to genes upregulated by *M. luteus* infection (Table 4.7), there were some similarities with those associated with genes upregulated upon *Ecc15* infection. The presence of terms related to transport, axon cargo transport and microtubule based transport, ion transport, ion homeostasis, exocytosis and chemical homeostasis are functionally similar to the GO terms enriched by *Ecc15* upregulated genes. However, in contrast to the limited enrichment observed by *Ecc15* upregulated genes, *M. luteus* upregulated genes demonstrated an enrichment of GO terms relating to the humoral immune response, in particular terms related to the defense response to Gram-positive bacterium, which in itself is not particularly surprising. Interestingly, genes upregulated upon *M. luteus* infection also exhibited enrichment for terms relating to tracheal network development, such as tube fusion (7) and branch fusion, open tracheal system (7), further suggesting that *M. luteus* may have a

direct impact on the development this system. Furthermore, 12 genes were associated with a term related to adult locomotory behavior, suggesting that *M. luteus* infection at the embryonic life stage may have a more long-term developmental impact on *Drosophila* development. A smaller number of biological process GO terms were enriched by genes downregulated upon *M. luteus* infection in the Stage 15 embryo (Table 4.8). Similar to those enriched by *Ecc15* downregulated genes, the majority of the GO terms enriched by genes downregulated upon *M. luteus* infection appear to relate to sensory processes, such as the detection of chemical stimuli. Nonetheless, a large number of genes (27) were also demonstrated to be associated with proteolytic functions.

To attempt to further derive information concerning the collective functions of up- and down-regulated genes upon *M. luteus* treatment within the Stage 15 embryo, STRING 9.0 was used to determine if any GO terms relating to molecular function and the cellular component concerned could be assigned to these genes. Numbers of genes associated with the each distinct GO term were counted and enrichment P-values where $p \leq 0.05$ were corrected for FDR, generating a q-value. Only those terms with a value of $q < 1.00$ were accepted. The molecular function GO terms associated with *M. luteus* upregulated genes are relatively extensive (Table 4.9), but not as diverse as those enriched by *Ecc15* infection. Nevertheless, the nature of molecular function GO terms associated with *M. luteus* upregulated genes corroborated the biological process GO. For example, many genes were associated with molecular function terms relating to voltage-gated ion channels and transmembrane transporters (Table 4.9), reflecting the enrichment of ion homeostasis and transport biological process GO terms (Table 4.7). Notably, genes were also associated with neurotransmitter release and neuropeptide Y receptor activity, suggesting the *M. luteus* infection may be able to alter neurotransmission within the Stage 15 embryo. However, as the q-values for these results are equivalent to 1, then these results should be considered as non-significant (Table 4.9). Conversely, genes downregulated by *M. luteus* infection (Table 4.10) were significantly attributed to a variety of molecular function GO terms relating to peptidase activity. Moreover, 6 genes were associated with

olfactory receptor activity, potentially shedding light on the enrichment of biological process GO terms relating to sensory processes and the detection of chemical stimuli (Table 4.8). However, other genes were associated with terms relating to structural functions, such as functioning as constituents of chitin-based cuticle, which does not necessarily corroborate any biological function GO terms associated with these genes.

In terms of the cellular localization of genes modulated upon *M. luteus* infection, large numbers of genes that displayed upregulation of expression upon *M. luteus* treatment were determined to have cytoplasmic, synaptic or reproductive tract cellular component ontologies (Table 4.11). Moreover, the more specific component cellular component GO terms identified would suggest that these genes may be integral in synaptic transmission and protein trafficking, relatively consistent with the biological processes and molecular function GO data (Tables 4.7 and 4.9). The cellular component GO term with the largest number of genes attributed (105) was highly general, relating to a cytoplasmic association, and from which it was difficult to attain any further functional relevance. Conversely, those genes that were downregulated upon *M. luteus* infection appear to be more highly enriched for extracellular regions or membranous components (Table 4.12), in contrast to the cytoplasmic associations of *M. luteus* upregulated genes.

Therefore, this data would collectively indicate that *M. luteus* infection induces genes associated with roles in humoral defense, ion transport and neurotransmitter release within the Stage 15 embryo.

Table 4.7: Biological process GO terms associated with genes upregulated upon *M. luteus* infection

GO Biological Process Term	Number of Genes (AU)	P-value	q-value
Single-organism transport	97	3.72E-07	1.95E-03
Transport	106	3.08E-06	8.80E-03
Establishment of localisation	107	1.22E-05	2.13E-02
Biological regulation	206	2.04E-05	2.68E-02
Axon cargo transport	7	3.15E-05	3.30E-02
Defense response to Gram-positive bacterium	10	6.66E-05	5.83E-02
Regulation of biological process	189	6.76E-05	5.07E-02
Regulation of membrane potential	7	6.95E-05	4.56E-02
Cellular ion homeostasis	12	7.28E-05	4.25E-02
Defense to Gram-positive bacterium	9	7.74E-05	4.06E-02
Antibacterial humoral response	8	8.10E-05	3.86E-02
Adult locomotory behaviour	12	8.69E-05	3.80E-02
Single-organism cellular process	272	8.69E-05	3.51E-02
Tube fusion	7	9.92E-05	3.72E-02
Branch fusion, open tracheal system	7	9.92E-05	3.47E-02
Cellular homeostasis	20	1.14E-04	3.74E-02
Cellular chemical homeostasis	12	1.44E-04	4.43E-02
Single organism process	328	1.93E-04	5.64E-02
Exocytosis	8	2.36E-04	6.51E-02
Ion transport	37	2.46E-04	6.45E-02
Ion homeostasis	12	2.66E-04	6.66E-02
Regulation of cellular process	174	2.71E-04	6.47E-02
Humoral Immune response	14	2.84E-04	6.48E-02
Antimicrobial humoral response	12	3.08E-04	6.74E-02
Defense response to	16	4.49E-04	9.43E-02

bacterium			
Cellular localisation	15	6.01E-04	1.21E-01
Chemical homeostasis	14	7.21E-04	1.40E-01
Single organism behaviour	34	8.75E-04	1.64E-01
Microtubule based transport	7	8.99E-04	1.63E-01

Table 4.8: Biological process GO terms associated with genes downregulated upon *M. luteus* infection

GO Biological Process Term	Number of Genes (AU)	P-value	q-value
Sensory perception of chemical stimulus	15	1.27E-04	2.46E-01
Proteolysis	27	1.38E-04	2.46E-01
Detection of chemical stimulus	9	1.44E-04	2.46E-01
Sensory perception	16	5.88E-04	7.56E-01
Puparial adhesion	3	1.06E-03	7.68E-01
Detection of stimulus	10	1.20E-03	7.68E-01

Table 4.9: Molecular function GO terms associated with genes upregulated upon *M. luteus* infection

GO Molecular Function Term	Number of Genes (AU)	P-value	q-value
CAAX-protein geranylgeranyltransferase activity	2	1.34E-03	1.00E+00
Chitin deacetylase activity	2	1.34E-03	1.00E+00
Kinesin binding	3	2.38E-03	1.00E+00
Sugar:hydrogen symporter activity	3	2.38E-03	1.00E+00
Monophenol monooxygenase activity	2	3.92E-03	1.00E+00
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, another compound as one donor, and incorporation of one atom of oxygen	2	3.92E-03	1.00E+00
Solute:hydrogen symporter activity	3	4.82E-03	1.00E+00
Purine ribonucleotide binding	44	7.75E-03	1.00E+00
Ribonucleotide binding	44	7.75E-03	1.00E+00
Purine ribonucleoside triphosphate binding	44	7.91E-03	1.00E+00
Purine nucleotide binding	44	8.41E-03	1.00E+00
Copper ion transmembrane transporter activity	2	1.24E-02	1.00E+00
Guanylate cyclase activity	3	1.31E-02	1.00E+00
Nucleotide binding	58	1.56E-02	1.00E+00
G-protein coupled peptide receptor activity	5	1.63E-02	1.00E+00
Neuropeptide receptor activity	5	1.63E-02	1.00E+00
G-protein coupled receptor activity	12	1.65E-02	1.00E+00
Peptide receptor activity	5	1.79E-02	1.00E+00
Protein geranylgeranyltransferase activity	2	1.82E-02	1.00E+00
Phosphotransferase activity, nitrogenous group as acceptor	2	1.82E-02	1.00E+00

Neuropeptide Y receptor activity	2	1.82E-02	1.00E+00
Glucose transmembrane transporter activity	3	1.91E-02	1.00E+00
Voltage-gated ion channel activity	5	2.16E-02	1.00E+00
Signal transducer activity	24	2.31E-02	1.00E+00
ATP binding	35	2.32E-02	1.00E+00
Adenyl ribonucleotide binding	35	2.42E-02	1.00E+00
Adenyl nucleotide binding	35	2.46E-02	1.00E+00
Protein prenyltransferase activity	2	2.49E-02	1.00E+00
Receptor activity	23	2.50E-02	1.00E+00
Neurotransmitter receptor activity	5	3.02E-02	1.00E+00
Hydrogen ion transporting ATP synthase activity, rotational mechanism	3	3.05E-02	1.00E+00
Hexose transmembrane transporter activity	3	3.05E-02	1.00E+00
Transmembrane transporter activity	36	3.06E-02	1.00E+00
Voltage-gated cation channel activity	4	3.45E-02	1.00E+00
Cation transmembrane transporter activity	21	3.93E-02	1.00E+00
Anion channel activity	3	3.97E-02	1.00E+00
Low-density lipoprotein receptor activity	2	4.06E-02	1.00E+00
Voltage-gated calcium channel activity	2	4.06E-02	1.00E+00
Cargo receptor activity	4	4.14E-02	1.00E+00
Monosaccharide transmembrane transporter activity	3	4.48E-02	1.00E+00
Small molecule binding	59	4.49E-02	1.00E+00
Monooxygenase activity	4	4.52E-02	1.00E+00

Table 4.10: Molecular function GO terms associated with genes downregulated upon *M. luteus* Infection

GO Molecular Function Term	Number of Genes (AU)	P-value	q-value
Peptidase activity, acting on L-amino acid peptides	29	4.42E-06	6.08E-03
Endopeptidase activity	25	6.35E-06	6.08E-03
Peptidase activity	29	8.76E-06	6.08E-03
Serine-type peptidase activity	19	1.17E-05	6.08E-03
Serine hydrolase activity	19	1.29E-05	6.08E-03
Serine-type endopeptidase activity	17	5.74E-05	2.26E-02
Olfactory receptor activity	6	1.25E-03	4.23E-01
Structural constituent of chitin-based cuticle	9	1.65E-03	4.86E-01
Structural constituent of cuticle	9	2.03E-03	5.32E-01
Transmembrane signaling receptor activity	15	2.92E-03	6.90E-01
Signaling receptor activity	15	4.47E-03	8.88E-01
Structural molecule activity	19	4.51E-03	8.88E-01
Aspartic-type endopeptidase activity	3	5.79E-03	9.77E-01

Table 4.11: Cellular component GO terms associated with genes upregulated upon *M. luteus* infection

GO Cellular Component Term	Number of Genes (AU)	P-value	q-value
Female germline ring canal outer rim	2	1.36E-03	6.30E-01
Synapse	14	1.63E-03	6.30E-01
Cytoplasm	105	2.19E-03	6.30E-01
Membrane-bounded vesicle	10	8.29E-03	6.30E-01

Table 4.12: Cellular component GO terms associated with genes downregulated upon *M. luteus* infection

GO Cellular Component Term	Number of genes (AU)	P-value	q-value
Extracellular region	25	6.77E-04	3.11E-01
Membrane part	44	7.21E-04	3.11E-01
Integral to membrane	34	2.99E-03	7.80E-01

4.2.5 Damage induced via sterile laser ablation significantly modulates expression in genes involved in the immune response, cuticle development and RNA splicing in the Stage 15 embryo

A large number of genes exhibited significant changes in expression within the Stage 15 embryo as a result of the sterile damage inflicted via laser ablation. These changes included both significant upregulation and downregulation of gene expression in ablated embryos compared to non-wounded controls. Further analysis and the correction of the P-values to account for false discovery rates (FDR) permitted the identification of 100 genes whose expression was most significantly modulated upon laser wounding in the Stage 15 embryo (Table A9.1, Appendix 9).

The genes undergoing the most significant changes in expression include members of the Tweedle (Twdl) gene family, which have been implicated in the determination of body shape and postulated to be integral to cuticle assembly (Guan *et al.,* 2006), with nine members (*TwdlY, TwdlV, TwdlK, TwdlM. TwdlQ, TwdlB, TwdlX, TwdlG* and *Twdlbeta*) experiencing significantly upregulated expression in the embryo upon laser ablation. Moreover, members of other gene families implicated in cuticle development were also shown to experience highly significant upregulation in expression upon wounding. These include 8 members of the Cuticular Protein (Cpr) family (*Cpr51A, Cpr62Bb, Cpr62Bc, Cpr65Av, Cpr50Cb, Cpr78Cc, Ccp84Ad* and *Cpr 56F*) and 3 members of the Larval Cuticle Protein (Lcp) family (*Lcp65Ae, Lcp65Ag3,* and *Lcp65Ad*). Other notable hits include a significant number of small nuclear RNA (snRNA) genes. The most significantly upregulated of these genes (*snRNA:U5:38ABb snRNA:U5:63BC, snRNA:U5:14B, snRNA:U5:38Aba, snRNA:U5:35D, snRNA:U5:23D, snRNA:U5:34A*) have all been previously implicated as functioning as components of the spliceosome, to enable intron splicing from mRNA transcripts (Chen *et al.,* 2005). A selection of genes encoding odorant-binding proteins (Obps) that mediate olfaction and chemoreception (Galindo and Smith, 2001; Zhou *et al.,* 2004) were also observed to exhibit highly significant upregulation upon sterile laser ablation of the Stage 15 embryo. However, many genes which were identified

as having highly significantly modulated expression upon wounding do not currently have any molecular or more general biological function assigned, and thus are highlighted in green in Table A9.1. These genes of unknown function encompass 41% of the 100 most significantly modulated genes, and include the four gene undergoing the most significant changes in expression: *CG13044, CG32548, CG42305* and *CG17325*. Interestingly, of the genes listed in Table A9.1, the vast majority of significant changes were due to an upregulation in expression; only 2 changes out of the most significant 100 are a result of downregulation and are indicated in red in Table A9.1. These genes are *mucin 30E* (*muc30E*), which encodes a component of extracellular matrix (Syed *et al.,* 2008), and *CG2901*, a gene postulated to play a role in synaptic target recognition (Kurusu *et al.,* 2008). Therefore, the predominately significant upregulation of these genes would hint at the existence of up-regulated gene regulatory networks in Stage 15 embryos which respond to damage stimuli.

In terms of genes previously annotated to play a role in the immune response to bacterial infection, only three are present in Table A9.1. These are *PGRP-LD*, *immune induced molecule 3* (*IM3*), which has been implicated in Toll pathway signaling and extension of lifespan (Bauer *et al.,* 2010), and *Pherokine-3* (*Phk-3*), a odour-binding protein which has been suggested to play a role in the neutralization of invading bacteria as well as tissue remodeling during metamorphosis (Sabatier *et al.,* 2003). Interestingly, *vago*, a gene which plays a crucial role in controlling viral load upon *Drosophila* C virus infection (Deddouche *et al.,* 2008), was also highly significantly induced within the Stage 15 embryos upon sterile wounding. Therefore, this provides further confirmation that damage alone is able to modulate the expression of the embryonic immune machinery and effectors. However, there is a conspicuous absence of AMP genes within Table A9.1, suggesting that modulation of AMP expression is not a primary response to damage. This is not to say that AMP genes are not significantly modulated upon wounding at all; 9 AMP genes demonstrated significant upregulation upon wounding (Figure 4.6; $p \leq 0.05$). These included *Att, Cec, Drs* and *Mtk* genes. Of these genes, *AttA, AttD* and *Dro5* were particularly highly induced by sterile laser ablation, suggesting that

there may be a preference for expression of these AMP isoforms upon damage stimuli within the Stage 15 embryo.

To further analyse the nature of the 100 most significantly modulated genes, gene ontology analyses focusing on biological process, molecular function and cellular component attributes were performed via the programmes GOrilla and STRING 9.0. This analysis identified 7 different ontological terms that could be associated with these genes, relating to their biological function (Table 4.13). These ontologies could be broadly sub-divided into those relating to mRNA processing and splicing, and those pertaining to cuticle development and body morphogenesis, further corroborating that sterile wounding activates gene networks that are involved in these processes. In particular, members of the Tweedle family of genes were attributed to ontological terms describing chitin-based cuticle development (Table 4.14). In addition to the contribution of the Tweedle family genes to this ontological category, the presence of *vermilion* (*v*), a gene encoding a tryptophan oxygenase (Baglioni, 1960; Baillie and Chovnick, 1971) which has been implicated in the control of tryptophan levels in the hemolymph in larvae and eye and cuticle pigmentation in the adult fly (Walker *et al.,* 1986), was also present.

When assigning gene ontology terms pertaining to molecular function, the resulting functional terms also reflected the importance of cuticle structure and integrity within the wounded Stage 15 embryo (Table 4.15). 72 genes were observed to be associated with terms related to the structural composition of the cuticle or more general structural functions. These findings were further corroborated by the assessment of cellular component ontology terms for the 100 most significantly modulated genes (Table 4.16), which elucidated that 49 of these genes encode products that are associated with the extracellular matrix or extracellular space. However, a further 3 genes were assigned more general molecular function terms pertaining to the inhibition of enzyme activity, and 2 were attributed fatty-acyl-CoA binding functions (Table 4.15), which may suggest roles in energy metabolism or lipid synthesis. Furthermore, 2 genes were associated with the microsome ontology term, which may reflect roles in protein synthesis for these particular genes. It could be speculated that

upregulation of genes involved in these processes may be required to meet the energy demands of repair processes within the embryo after wounding.

Subsequently, analysis using STRING to detect known and theorized protein-protein interactions was performed on the list of candidate genes in Table A9.1; to identify any potential gene networks that respond to damage within the Stage 15 embryo (Figure 4.7). Considering the 100 most significantly modulated wound-responsive genes, one large network of potential protein-protein interactions was proposed, with three sets of smaller potential interactions also identified. Most of the proposed interactions derive from evidence of co-expression or co-occurrence, as demonstrated by the black and blue edges respectively, with fewer interactions proposed on the basis of experimental evidence or collective citation in scientific literature, represented by pink and green edges respectively. The large network itself consisted of many members of the Twdl and Cpr family members, suggesting that this central network may regulate cuticle development post wounding. However, not all members of the Twdl family are included within this network; only *TwdlB, Twdlbeta, TwdlV, TwdlM* and *TwdlG* occupy central positions, with all other family members excluded from any network. This may suggest that only these 5 family members are central to mediating the cuticle response to damage within the embryo.

It is also of interest that this cuticlar network also incorporates a number of genes whose molecular function and biological relevance remains unknown, which may indicate that these genes may have a role in *Drosophila* cuticle repair, maintenance or development. Table 4.17 displays a list of unannotated genes that are inferred to interact with at least one member of the Twdl or Cpr families. The probability of these interactions representing actual biological interactions was calculated by STRING in accordance with von Mering *et al.* (2005). The majority of the listed unannotated genes appear to have predicted interactions with between 3 to 5 Twdl or Cpr family members, with the exceptions of *CG16885* and *CG14752* which are only predicted to interact with *Cpr62Bc*. Furthermore, the probability values that these predictions represent actual functional interactions range from 0.423 to 0.991, inferring that it is

probable that many of these proposed interactions have a probable functional basis. Of particular note are the proposed interactions between *CG14147* and *TwdlB*, and *CG14752* and *Cpr62Bc*, which have greater than 90% probability of representing actual biological interactions. Thus, it is highly probable that these two previously unannotated genes are not only highly significantly wound responsive, but also function in conjunction with Twdl and Cpr family members to mediate a cuticular response to damage.

Of the other interactions that are independent of the central cuticular network, the most interesting are those relating to *IM3*. This network consists of three nodes (*IM3, CG17325* and *CG6870*) and two green edges, indicating that the proposed interactions are based upon co-citation within the scientific literature. The interaction between *IM3* and *CG17325* is notable, as the latter was observed to experience the fourth most significant upregulation of expression within the entire dataset. Thus, this proposed interaction with IM3 may suggest an immune function for *CG17325*. Moreover, upregulation in expression of all three of these candidate genes has previously been associated with increases in life span (Bauer *et al*., 2010), thus potentially suggesting interplay between damage and the resulting lifespan of the affected embryo. However, the probabilities that these predicted functional interactions exist are only 49% and 55% respectively.

Figure 4.6: AMP gene expression is significantly up-regulated upon Sterile Laser Ablation of Stage 15 Embryos

Comparison of the non-wounded and sterile laser ablated Stage 15 embryo transcriptomes elucidated that 9 AMP genes were significantly upregulated in wounded embryos ($p \leq 0.05$). These included members of the *Att, Cec, Drs* and *Mtk* classes. The upregulation of *AttA, AttD* and *Dro5* was particularly prominent, suggesting a preferential expression of these AMPs is mediated upon damage stimuli within the Stage 15 embryo.

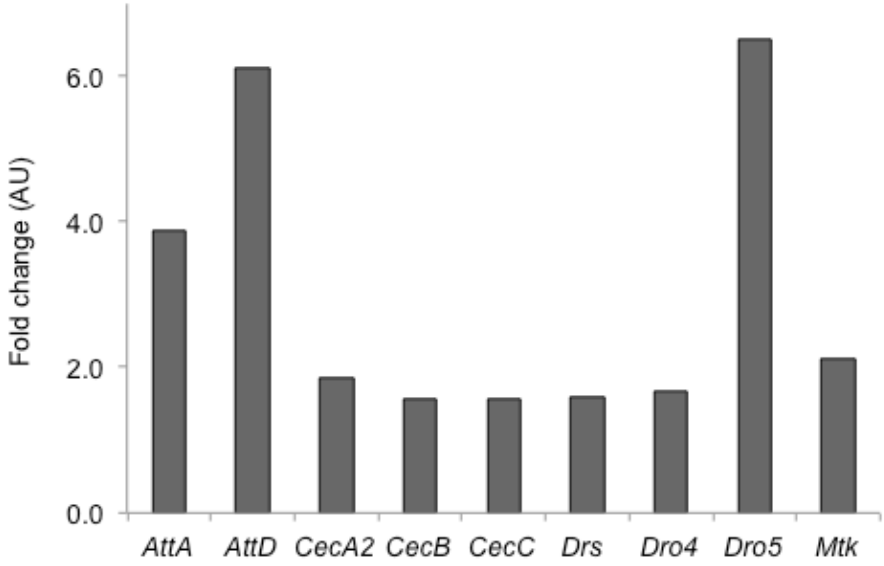

Fold change (AU)
6.0
4.0
2.0
0.0
AttA
AttD
CecA2
CecB
CecC
Drs
Dro4
Dro5
Mtk

Table 4.13: Biological Process GO of Wound Modulated Genes

Gene ontology description	P value	Number of genes
Body morphogenesis	1.84E-10	8
Cuticle development	2.81E-07	10
Chitin-based cuticle development	3.00E-07	9
RNA splicing, via transesterification	2.12E-02	8
RNA splicing	2.37E-02	8
RNA splicing via transesterification with bulged adenosine as nucleophile	3.09E-02	8
mRNA splicing, via spliceosome	3.09E-02	8

Table 4.14: Chitin-based cuticle development genes modulated upon wounding

CG number	Gene name	Gene ontology function
CG14250	*TwdlQ*	Chitin-based cuticle development
CG14640	*TwdlV*	Chitin-based cuticle development
CG14643	*TwdlG*	Chitin-based cuticle development
CG32570	*TwdlY*	Chitin-based cuticle development
CG32571	*TwdlX*	Chitin-based cuticle development
CG3757	*v*	Cuticle pigmentation Regulation of adult chitin-containing cuticle pigmentation
CG5469	*TwdlM*	Chitin-based cuticle development
CG6460	*TwdlK*	Chitin-based cuticle development
CG6478	*TwdlB*	Chitin-based cuticle development
CG8986	*TwdlBeta*	Chitin-based cuticle development

Table 4.15: Molecular Function GO terms associated with Wound Modulated Genes

Gene ontology description	Number of genes	P-value
Structural constituent of chitin-based cuticle	21	3.39E-27
Structural constituent of cuticle	21	6.66E-27
Structural molecule activity	24	4.14E-19
Structural constituent of chitin-based larval cuticle	6	3.62E-08
Fatty-acyl-CoA binding	2	8.91E-04
Enzyme inhibitor activity	3	1.96E-02

Table 4.16: Cellular Component GO terms associated with Wound Modulated Genes

Gene ontology description	Number of genes	P-value
Extracellular matrix	10	6.45E-13
Proteinaceous extracellular matrix	9	9.87E-13
Extracellular region	18	2.75E-12
Extracellular region part	12	5.46E-09
Microsome	2	4.05E-02

Figure 4.7: Network analysis of the most significantly modulated genes upon sterile laser ablation of the Stage 15 embryo

STRING 9.0 was utilized to predict networks of genes that may be upregulated upon damage stimuli in the Stage 15 embryo. Nodes represent proteins products encoded by the 100 most significantly modulated genes within the Stage 15 embryo upon sterile laser ablation. Black edges reflect predicted interactions based on co-expression evidence. Pink edges represent interactions predicted on experimental evidence. Blue edges reflect proposed interactions based on evidence of co-occurrence of protein products. Green edges are interactions that are predicted on text-mining evidence. One large central network was observed, which was dominated by members of the Twdl and Cpr families, although also incorporated previously unannotated genes and thus suggesting potential roles for these genes in cuticule development.

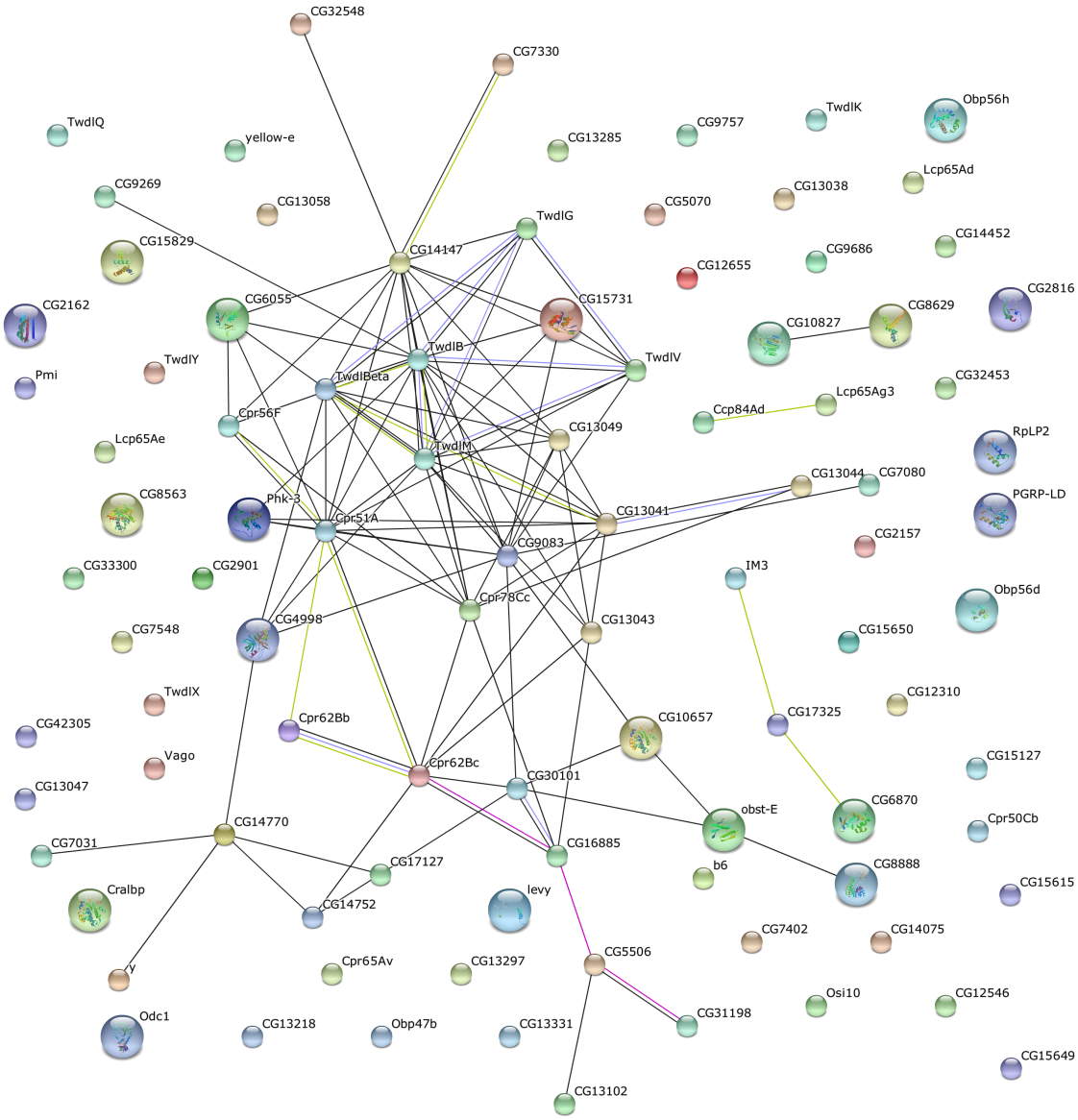

CG32548
CG7330
TwdlQ
yellow-e
CG13285
CG9757
TwdlK
Obp56h
CG9269
CG13058
CG5070
CG13038
Lcp65Ad
CG15829
TwdlG
CG14147
CG12655
CG9686
CG14452
CG2162
CG6055
CG15731
CG10827
CG8629
CG2816
TwdlB
TwdlY
TwdlV
Pmi
TwdlBeta
CG32453
Lcp65Ag3
Ccp84Ad
Cpr56F
CG13049
RpLP2
Lcp65Ae
TwdlM
CG13044
CG7080
CG8563
Phk-3
Cpr51A
CG13041
PGRP-LD
CG9083
CG2157
CG33300
CG2901
IM3
Cpr78Cc
Obp56d
CG4998
CG13043
CG7548
CG15650
TwdlX
CG12310
CG42305
Cpr62Bb
CG10657
CG17325
Vago
Cpr62Bc
CG30101
CG15127
CG13047
obst-E
CG6870
CG14770
Cpr50Cb
CG7031
CG16885
CG17127
b6
CG8888
CG15615
Cralbp
levy
CG14752
CG7402
CG14075
CG5506
Cpr65Av
CG13297
Osi10
CG12546
CG31198
Odc1
CG13218
Obp47b
CG13331
CG15649
CG13102

Table 4.17: Interactions between Unannotated Genes and Genes with Annotated Cuticular Functions located within the proposed cuticular network

Unannotated gene name	Interacting cuticular gene name	Probability of interaction with Twdl or Cpr family member (AU)
CG14147	*TwdlB*	0.915
	TwdlBeta	0.887
	TwdlV	0.811
	Cpr56F	0.736
	Cpr51A	0.804
CG15731	*TwdlV*	0.621
	TwdlB	0.872
	TwdlM	0.750
CG13041	*TwdlB*	0.871
	TwdlBeta	0.423
	TwdlM	0.881
	Cpr78Cc	0.685
	Cpr62Bc	0.445
CG9083	*Twdl B*	0.778
	TwdlM	0.669
	Cpr51A	0.597
CG13043	*TwdlB*	0.627
	TwdlM	0.591
	Cpr62Bc	0.475
CG16885	*Cpr62Bc*	0.991
CG14752	*Cpr62Bc*	0.597

Red values highlight results that infer >90% chance that predicted protein-protein interactions are true positive results.

4.2.6 The embryonic transcriptome upon PBS injection shares similarities to that induced upon sterile laser ablation and bacterial treatment

To compare the effects of different types of sterile damage on the immune response of the *Drosophila* embryo, Stage 15 embryos were injected with PBS, alongside naïve controls and the transcriptional profiles of these embryos obtained via microarray studies. Comparison between the naïve and PBS injected transcriptomes (n=3) would then allow for identification of genes significantly modulated by this type of damage. Analysis of the resulting data permitted the identification of the 100 genes showing the greatest log fold up- or downregulation upon PBS treatment (Tables A10.1 and A10.2 respectively, Appendix 10). Due to variation in the samples, no genes were demonstrated to be significantly upregulated upon PBS treatment within the Stage 15 embryo, thus results should be viewed with caution.

Considering the top 100 genes upregulated in the Stage 15 embryo upon PBS treatment (Table A10.1), many genes involved in cuticle development (28) were shown to be upregulated, as in the case of sterile laser ablation treatment. This included *drop dead* (*drd*), *Obstructor-E* (*obst-E*), members of the Twdl family, such as *TwdlB*, *TwdlBeta*, *TwdlK* and *TwdlM*, Cpr family members, such as *Cpr100A*, *Cpr6Bc* and *Cpr65Ea*, and Lcp family members, such as *Lcp65Ac*, *Lcp65Ae* and *LcpAg1*. Interestingly, other Twdl family members were induced upon sterile PBS injection that were not significantly induced by sterile laser ablation. These included *TwdlC, TwdlD, TwdlL, TwdlN* and *TwdlP*. This may indicate that different types of sterile damage may initiate differential cuticular responses within the Stage 15 embryo. More broadly, it would suggest that cuticle repair is an essential embryonic response to damage, regardless of the precise nature of damage or the method of its infliction.

In terms of the genes downregulated upon PBS treatment of Stage 15 embryos (Table A10.2), the range of gene functions was much greater. Two Cpr family members (*Cpr47Ef* and *Cpr65Az*) were downregulated upon PBS treatment, suggesting that the cuticlar response to damage is more complex

then simply an upregulatory gene network. Other genes involved in dorsal-ventral axis specification (*pipe, pip*; *shavenoid; sha*), synaptic growth (*highwire, hiw*) and cell proliferation (*liquid-facets related; lqfR*). Moreover genes involved in ecdysone signaling were downregulated upon this form of sterile damage, such as *Eip71CD* and *shd,* concurrent with previous results (Chapter 3). Thus, the observed range of genes downregulated upon damage via sterile PBS injection was broader, and may reflect the downregulation of processes which are not necessarily required for wound healing.

Comparison between the top 100 genes modulated by PBS injection and those modulated by *Ecc15* (OD=1) and M. luteus (OD=1) infection was also performed; to determine the relative proportion of genes which may be modulated by both damage and infection. Transcriptomes of *Ecc15, M. luteus* or PBS injected embryos were compared to those of naïve embryos (n=3), and the top 100 genes up- or downregulated upon each of these treatments were selected and the content compared (Figure 4.8). These comparisons demonstrated that 56% of the top 100 upregulated genes were common to all three treatments (Figure 4.8A), suggesting that the majority of most highly upregulated genes in the Stage 15 embryos upon these treatments are due to sterile damage, and not bacterial infection itself. However, it was also interesting to note that further subsets of upregulated genes were only shared between the Gram-positive or Gram-negative infected and PBS injected transcriptomes; with 12% and 9% of genes shared between *Ecc15* and PBS injected and M. luteus and PBS injected embryos respectively. A similar trend was noted within the top 100 genes downregulated upon these treatments (Figure 4.8B). In this case, however, the overlap of genes was much smaller, with only 26% of genes showing downregulation upon all three treatments. Moreover, the proportion of overlap between PBS and *Ecc15* downregulated genes was much lower, with only 1% of genes downregulated by both treatments. Conversely, PBS and *M. luteus* downregulated genes demonstrated a 17% overlap; an increase proportion compared to that of the upregulated genes. Taken together, this could suggest that these two bacterial species may have their own specific interactions with the damage response, leading to the stimulation and repression of individual gene targets. This

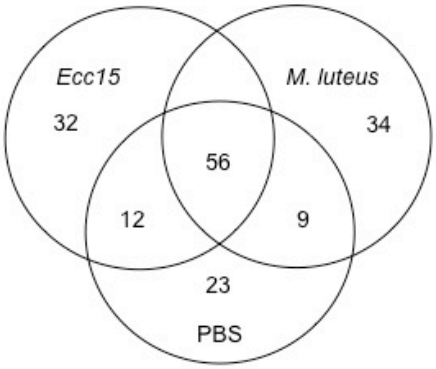
observation could be further substantiated by the fact that some genes appeared to be upregulated or downregulated solely upon PBS injection alone, and not by either injection of *Ecc15* or *M. luteus* (Figure 4.8C); permitting the identification of genes which may be crucial to the damage response alone. These include structural components of the chitin cuticle (*Tubby, Tb; Cpr65Ec*), *Tetraspanin 42Er* (*Tsp42Er*), Cytochrome P450 enzymes (*Cyp4AD1),* genes involved in cell adhesion and tissue regeneration (*Ninjurin A; nijA*), and odorant binding proteins (*Obp47b).*

Therefore, damage inflicted by sterile PBS injection of Stage 15 embryos upregulated many families of genes which were also activated by sterile laser ablation, indicating that the broad damage response within the *Drosophila* embryo is mediated irrespective of the nature of damage. However, the fact that different family members were induced upon sterile laser ablation and sterile PBS injection may indicate that there is a greater degree of specificity in the damage response itself. Furthermore, comparison of PBS and bacterial infected embryo transcriptomes eludicated that a large proportion of genes modulated upon these treatments may be due to damage, although subsets of bacteria-specific and damage-specific genes can be identified from these datasets.

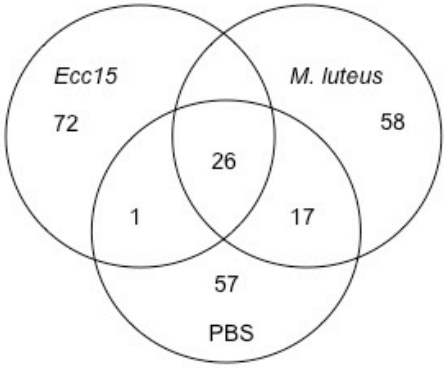
Figure 4.8: Comparison of the top 100 upregulated and downregulated genes upon PBS, *Ecc15* and *M. luteus* treatment in Stage 15 embryos.

(**A-B**) The top 100 genes that were observed to be modulated upon PBS, *Ecc15* (OD=1) or *M. luteus* injection (OD=1) were compared to determine the potential overlap of gene expression upon these treatments, and to determine if any damage specific genes could be identified. Results were normalized to naïve transcript levels for these analyses (n=3). In the lists of the top 100 upregulated genes for each treatment (**A**), 56% of genes upregulated by PBS injection were also upregulated in both *Ecc15* and *M. luteus* treated embryos. 12% and 9% of genes upregulated by PBS injection were observed to overlap with *Ecc15* and *M. luteus* upregulated genes respectively. The top 100 downregulated genes for each treatment showed a similar trend (**B**), but to a lesser degree; with 26% of genes downregulated in all three treatments, 1% downregulated by both PBS and *Ecc15* treatment and 17% downregulated by both PBS and *M. luteus* treatment. These comparisons also identified subsets of genes which were modulated by PBS injection alone (**C**), and hence may be considered as damage-specific genes.

A



B



C

Upregulated	Downregulated
Ccp84Ae	*Abl*
CG10472	*Art4*
CG10911	*bol*
CG11159	*CG10107*
CG13026	*CG10348*
CG13060	*CG11029*
CG13217	*CG12009*
CG13314	*CG12050*
CG13618	*CG13096*
CG14205	*CG13622*
CG18294	*CG14803*
CG18649	*CG16798*
CG30101	*CG17549*
CG32829	*CG17672*
CG4962	*CG17780*
CG5326	*CG18810*
CG6277	*CG3108*
Cpr65Ec	*CG31244*
Drd	*CG32642*
Lcp65Af	*CG32643*
Obp47b	*CG34350*
Tb	*CG4164*
Tsp42Er	*CG42542*
	CG4558
	CG4702
	CG5150
	CG5776
	CG6118
	CG6133
	CG8878
	CG9143
	CG9592
	CG9993
	Cpr47Ef
	Cpr65Az
	CR10102

	Csat
	Cyp4ad1
	Eip71CD
	fbl6
	GalNAc-T2
	glo
	hiw
	ImpE2
	lqfR
	Lsp2
	mei-217
	NijA
	Orc1
	Patj
	Sas10
	shd
	Snm1
	spag4
	Wnt2
	Wnt4

4.2.7 Analysis of hemocyte transcriptional profiles upon infection and damage stimuli reveals modulation of genes involved in oxidation-reduction, transcriptional regulation and tracheal development, but also a lack of potential hemocyte-specific gene modulatory networks

To more closely assess the contribution of hemocytes to the total immune response of the *Drosophila* embryo, Stage 15 embryos expressing GFP specifically within hemocytes using the Gal4-UAS system were injected either with sterile endotoxin-free PBS or *Ecc15* (OD=1). The hemocytes from these embryos were subsequently isolated by FACS, alongside those from equivalent naïve controls. The RNA was extracted from the sorted hemocytes and utilized in preliminary microarray studies (n=1), to examine the transcriptional profiles of hemocytes upon infection with *Ecc15* and mechanical damage induced via PBS injection. Analysis of the resulting data from these experiments permitted the identification of the 100 genes showing the greatest log fold up or downregulation upon either *Ecc15* or PBS treatment (Tables A11.1, A11.2, A12.1, A12.2; Appendices 11 and 12).

The 100 most highly up- and down-regulated genes within hemocytes upon *Ecc15* infection can be viewed in Tables A11.1 and A11.2 respectively. Considering the 100 most upregulated genes within hemocytes upon *Ecc15*, the highest fold change in expression (11.454-log fold) was noted in the *Ionotropic receptor 41a* (*Ir41a*), which plays a role as an olfactory receptor and has been shown to be derived from ionotropic glutamate receptors (Benton *et al.,* 2009; Croset *et al.,* 2010). Other notable hits include genes involved in the oxidation-reduction process, such as the oxidoreductase *ninaG*, *Cyp*4p1 and *Cyp4d8.* Notably, *Cyp4p1* and *Cyp4d8* have also been demonstrated to possess iron and heme binding properties (Flybase Curators, 2004). Further genes were observed to encode proteins involved in transcription and mRNA processing, such as the post-translational gene silencer *argonaute 3* (*AGO3*), *Nuclear export factor 3* (*Nfx3*), the RNA metabolism regulator *Antennapedia* (*Antp*) and the regulators of transcription *Forkhead Domain 96Ca* (*fd46Ca*), *poils au dos* (*pad*) and *sisterless A* (*sisA*). However, upregulation of genes implicated in protein transport, such as *Rab9Db*, and proteolysis, such as

Pepsinogen-like (*Pcl*), *tolloid* (*tld*) and *Nep5*, was also observed. Interestingly, upregulation of genes such as *dead end* (*dnd*) and *branchless* (*bnl)*, which are required for tracheal branch fusion (Jiang *et al.,* 2007; Kakihara *et al.,* 2008) and morphogenesis of branching epithelia (Affolter and Shilo, 2000) as part of tracheal development respectively, may suggest an interplay between the tracheal network and hemocytes upon *Ecc15* infection. Likewise, the hemocyte upregulation of *Rfx* and *Ndc8*0 may be indicative of interaction with the embryonic nervous system upon *Ecc15* infection, due to their observed roles in neurogenesis (Dubruille *et al.,* 2002; Neumuller *et al.,* 2011). Other highly upregulated genes have previously been implicated in cell adhesion (*brother of ihog, boi*), the stress response and life-span (*methuselah-like 9, mthl9*), actin filament organization (*RhoGAP18B*), anion transport (*Bestotrophin 1, Best 1; Organic anion transporting polypeptide, Oatp33Eb*) and DNA double-strand break processing (*Meiotic-W8, Mei-W8*). Furthermore, when assessing the 100 genes experiencing the greatest downregulation in hemocytes upon *Ecc15* infection (-4.284 to -6.619 log fold-change; Table 5.22), some findings clearly complement the upregulatory response. For instance, downregulation of *bru-2*, a negative regulator or translation, and *ss,* a negative regulator of transcription, is consistent with the upregulation of genes involved in mRNA processing and transcription. Moreover, the downregulation of *Serpin43Ab* (*Spn43Ab*) is concordant with large increases in serine endopeptidase gene expression observed. Other hits of interest include downregulation of *gless*, which has been implicated in the cellular response to stress and as a negative regulator of apoptosis (Grant et *al.,* 2010). However, it is also notable that not all results would be indicative of an effective hemocyte immune response. For example, downregulation of *inscuteable* (*insc*), which plays a role in survival to bacterial infection (Berkey *et al.,* 2009), was observed, alongside substantial decreases in expression *Hormone Receptor-like in 39* (*Hr39*), a 20-HE signaling target gene implicated in autophagic cell death (Takemoto *et al.,* 2007). Of particular interest is the downregulation of *wing blisters* (*wb*) and *pole hole* (*phl*), whose expression is required for correct tracheal development (Martin *et al.,* 1999; Jiang and Edgar, 2009), as well as *ZAP3* and *extra-extra* (*exex*), which are genes required for neuron projection morphogenesis and central nervous system

development respectively (Odden and Doe, 2001; Broihier and Skeath, 2002; Sepp *et al.,* 2008). However, the downregulation of these genes within hemocytes is also seemingly in contradiction with the nature of genes upregulated in hemocytes upon *Ecc15* infection. Moreover, downregulation of genes implicated in hemocyte maintenance and proliferation were also observed; for instance, *Phl*, also required for the survival of circulating hemocytes in *Drosophila* (Sackton *et al.,* 2010) and *couch potato* (*cpo*), a regulator of hemocyte proliferation (Sinenko *et al.,* 2010), were highly downregulated in hemocytes upon *Ecc15* infection, potentially contraindicating the requirements of the embryo host and enabling *Ecc15* to proliferate unchecked.

The genes most highly modulated upon damage induced by PBS injection (Table A12.1, Appendix 12) displayed similar trends in terms of broad function to those modulated by *Ecc15* infection. For example, many genes involved in the oxidation-reduction process were highly upregulated, including *laccase2*, *Cyp6t1, Cyp4t1, Cyp316a1* and *peroxidase* (*pxd*). Numerous members of the Cpr family of genes (*Cpr97Ea, Cpr66Cb, Cpr64Aa*) were also upregulated in hemocytes, possibly suggestive of a role in the repair of damaged cuticle following the injection process. Other hits of interest encompassed genes with function in phagocytosis (*pxd*), macroautophagy (*Buffy*), actin polymerization (*Verprolin 1, Vrp1; Diaphanous, Dia*), 20-HE biosynthesis (*neverland, nvd*), nervous system development (*minibrain, mnb; nubbin, nub*) and calcium binding (*Troponin C at 41C, TrpC41C*). Of notable interest was the up-regulation of *PGRP-LB*, a negative regulator of IMD signaling (Zaidman-Remy *et al.,* 2006), and *lozenge* (*lz*), a gene required for effective wound healing in *Drosophila* larvae (Galko and Krasnow, 2004). Similar to the nature of genes observed to be downregulated in hemocytes upon *Ecc15* treatment, negative regulators of transcription, such as *scratch* (*scrt*) and net, were observed to be downregulated upon PBS treatment (Table A12.2, Appendix 12); this may be suggestive of a mechanism to maintain the increased levels of transcription required by hemocytes to mediate an effective response to damage. Interestingly, in addition to those observed to be upregulated upon PBS treatment, some genes involved in the oxidation-reduction process were

observed to be downregulated upon injection-induced damage; these included *Protoporphryiogen oxidase* (*Ppox*) and *puglist* (*pug*). Also of note was the down-regulation of genes involved in the defense response to bacterial infection, such as Heat Shock Factor (*Hsf*), *Dorsal Interacting Protein 3* (*Dip3*) and *Taspase 3*, and genes implicated in the DNA damage response, including *Xeroderma pigmentosum D ortholog* (*Xpd*). Therefore, the hemocyte transcriptional responses to damage and infection show a degree of similarity, in that they involve modulation of genes that play roles in some similar biological processes, although there appears to be a distinct division of genes that are modulated by each of these different stimuli. Moreover, there also appears to be a division in the modulation of genes with functions in the same biological processes; with some genes experiencing up-regulation and other experiencing down-regulation upon the same stimuli.

Intriguingly, of the top 100 genes up-regulated in hemocytes upon *Ecc15* infection or PBS injection, only 11% were observed to be commonly upregulated by both treatments (Figure 4.9A). These include genes encoding the 20-HE biosynthetic enzyme phantom (phm), the serine endopeptidase Corin, the regulator of myoblast fusion M-spondin (mspo), Phosphodiesterase 1c (PDE1c) and the oxidoreductase CG33093 (Figure 4.9B). A similar trend was noted for the most highly downregulated genes in hemocytes upon *Ecc15* or PBS injection; there was 7% overlap in the top 100 hits (Figure 4.9A). These common genes encode proteins implicated in lipid and carbohydrate metabolism (CG31414, CG31871), neurogenesis (CG42588), tryptophanyl-tRNA aminoacylation (CG7441) and actin filament organization or regulation of cell shape (CG7497) (Figure 4.9B). Therefore, *Ecc15 i*nfection and damage promote highly disparate profiles of transcriptional modulation within hemocytes, in terms of the 100 genes most highly modulated upon these treatments, with relatively little overlap. Where overlap of gene modulation exists, this would suggest that these genes are not specific infection-responsive genes, since the damage induced via PBS injection is also able to highly stimulate their expression. Thus, these common genes represent potential damage-responsive gene targets, contributing to biological processes that may be regulated by damage stimuli.

STRING analysis to detect known and theorized gene-gene/protein-protein interactions was employed on the lists of candidate genes in Tables A11.1, A11.2, A12.1 and A12.2; to identify any potential gene networks that are responsive to infection and damage stimuli within hemocytes. Surprisingly, despite the large fold-changes observed, very few interactions appear to exist between the 100 most modulated gene products upon both *Ecc15* and PBS injection in hemocytes. For instance, considering firstly the network analysis of those genes upregulated in hemocytes by *Ecc15* infection (Figure 4.10), there are only 4 predicted interactions. These are between *Phosphodiesterase 1c* (*PDE1c*) and *Adenylate cyclase 3* (*Ac3*), *Cardioacceleratory peptide* (*Ccacp*) and *CG11475,* and *CG18371*, a gene encoding a protein with proposed acylphosphatase activity (Flybase Curators, 2004), with both *CG3085* and *CG9406*, which have been implicated in microtubule organization and calcium ion binding respectively (Flybase Curators, 2004). Moreover, none of these predictions have a high probability of reflecting true protein-protein interactions (Figure 4.10), with the exception of that concerning *PDE1c* and *Ac3*. A similar lack of interactions was predicted between the genes most significantly downregulated upon *Ecc15* infection in hemocytes (Figure 4.11). 9 interactions were predicted over 4 separate networks, with the probability score for the majority of these predictions being relatively low. The largest downregulatory network observed consists of 5 nodes and 5 edges, constructed on the evidence of co-expression data. *CG14814*, a gene of unknown function, is the central node, with predicted interactions with genes involved in spindle assembly (*dgt5* and *Cks30A*), a gene with predicted transposase activity (*CG4570*), and a gene of unknown function, which may also interact with *Cks30A* (*CG8924*). Thus, *CG8924* and *CG14814* may potentially play roles in cell division and may be co-downregulated upon *Ecc15* infection in hemocytes. Interestingly, other minor predicted downregulatory networks identified implied co-regulation of genes involved in DNA repair (*Rad9, Spindle-B* (*spn-B*) and *CG30169*), and in cation transport (*Shaker* (*Sh*) and *Small Conductance Calcium-activated potassium channel* (*SK*)). Therefore, STRING analysis of the 100 genes which demonstrated the greatest up or downregulation upon *Ecc15* infection in hemocytes would suggest an apparent

lack of interactions between their resulting protein products, and hence would infer an absence of either upregulatory or downregulatory gene networks that respond to *Ecc15* infection in hemocytes.

However, this effect is not limited to *Ecc15* treatment, as hemocyte transcription profiles upon PBS injections also display a stark absence of potential interactions. The resulting predicted networks of the 100 most highly upregulated genes in hemocytes upon PBS injection are relatively few and simplistic in nature (Figure 4.12). The largest predicted interaction network consists of 4 nodes and 4 edges, the cornerstone of which is *Tsp42Ec*, an uncharacterized tetraspanin-encoding gene. Of the other genes in this network, the functions of *CG15423* and *CG4377* are unknown and that of *CG3987* remains relatively undefined, although there is evidence to suggest that upregulation of this gene may be important in the response to parasitisation (Roxstrom-Lindquist *et al.,* 2004) and in mesoderm development (Furlong *et al.,* 2001). A predicted interaction between *CG33093* and *CG15829* further highlights the importance of oxidation-reduction reactions upon PBS injection, as these genes are proposed to play roles within this process (Flybase, 1992; Flybase Curators, 2004). Moreover, the proposed network between *Zwilch*, a gene implicated in mitosis (Williams *et al.,* 2003), and two genes of unknown function (*CG11120* and *CG9023*), may further highlight the requirement for new cells to replace those damaged in response to ROS generated by the damage incurred from injection, as well as permitting the preliminary functional annotation of two previously uncharacterized genes. Conversely, greater numbers of the most highly downregulated genes within hemocytes upon PBS injection form a much more coherent interactive network (Figure 4.13), consisting of 12 nodes and 11 edges, representing interactions via a combination of co-expression, co-occurrence, experimental, database and literature evidence. Moreover, many of the interactions within this central network are predicted to be highly probable, as highlighted in red in Figure 4.13. For example, the interactions between the central elements of the network (*RpI135, Xpd, Tbp* and *CG9305*) are all estimated to have a probability score of ≥90%. These aforementioned genes play roles within gene transcription within *Drosophila* systems, either as subunits of RNA polymerase

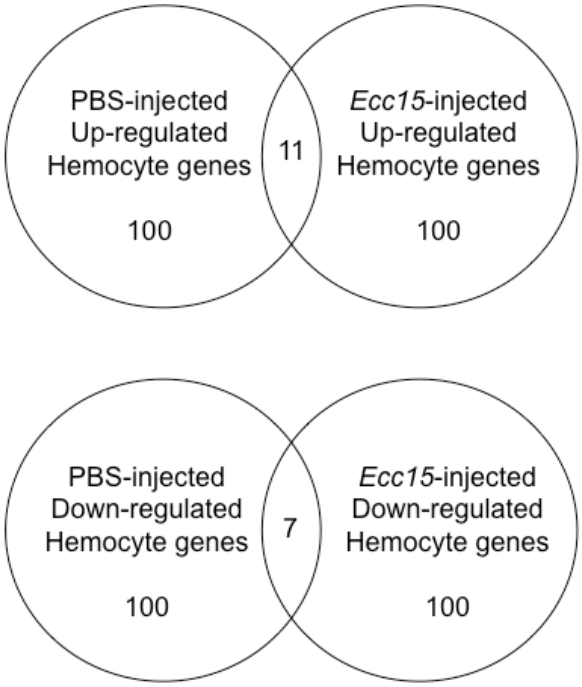
l (*Rpl135*), or transcription factors (*Xpd* and *Tbp*). Other genes within this central network, such as *Hsf, CG9630* and *Trf* also have functions within transcriptional processes (Hansen *et al.,* 1997; Chen *et al.,* 2009; Takeda *et al.,* 2000), inferring that this structure may represent a downregulatory network to modulate transcription. Another highly probable interaction was that between *synaptojanin* (*synj*) and the *inositol 1-2C4-2C5-2C-trisphosphate receptor* (*Itp-r38A*), suggesting a role for the downregulation of the phosphatidylinositol signaling system upon damage signaling.

Taken together, these results suggest that the hemocyte transcriptional response to damage and *Ecc15* infection is highly diverse, potentially contributing to an array of different biological processes. Whilst there is a limited degree of overlap between modulated genes within these responses and the biological processes that they potentially mediate, the transcriptional profiles of the hemocyte upon damage or infection were distinct. However, the apparent lack of hemocyte upregulatory or downregulatory transcriptional networks may imply that genes within hemocytes are modulated in individually or in small networks, or within networks which have not been detected by STRING.

Figure 4.9: Top 100 genes commonly up- and down-regulated in hemocytes upon *Ecc15* infection and PBS injection

(A-B) Comparison of genes commonly modulated in hemocytes upon *Ecc15* or PBS treatment. (A) Of the top 100 most significantly up- or down-regulated genes in hemocytes upon *Ecc15* or PBS injection, only 11% and 7% were commonly up or down-regulated respectively, suggesting relatively distinct responses by hemocytes to damage and infection. (B) List of genes commonly upregulated or downregulated in hemocytes by *Ecc15* and PBS treatments.

A

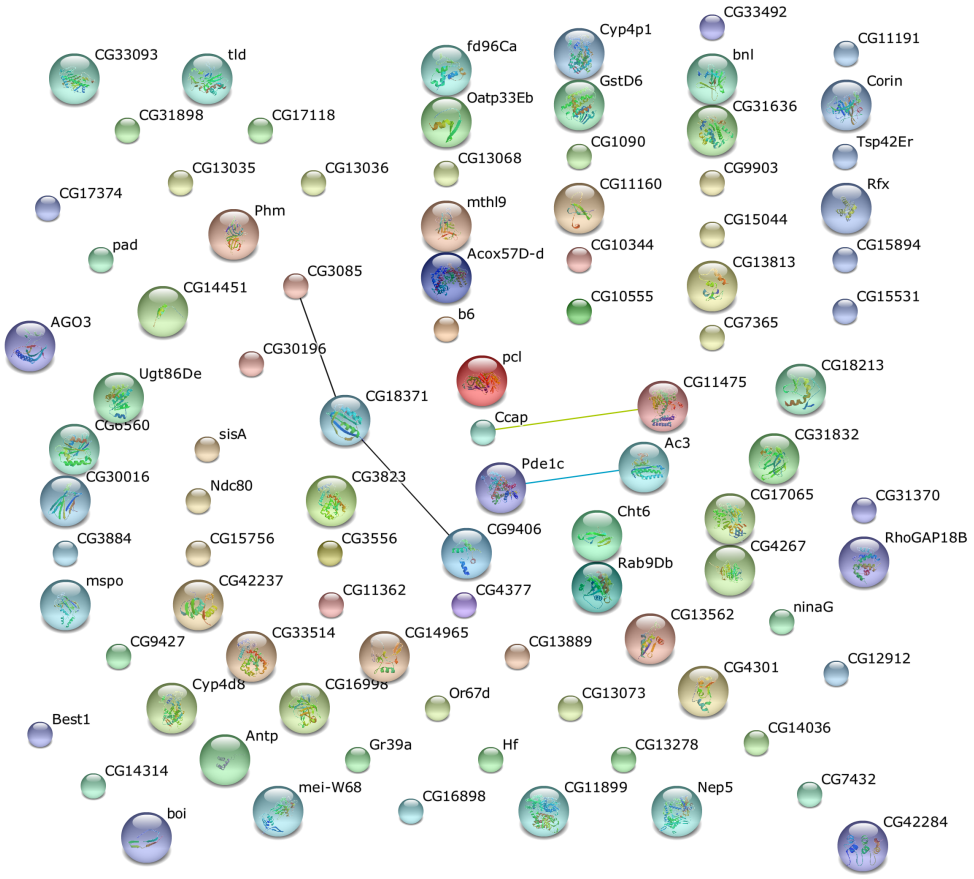


B

Up-regulated hemocyte genes common to PBS and Ecc15 injection	**Down-regulated hemocyte genes common to PBS and Ecc15 injection**
CG10344	*CG16868*
CG31370	*CG31414*
CG33093	*CG31871*
CG42797	*CG42588*
CG43337	*CG4741*
Corin	*CG7441*
Cyp4p1	*CG7497*
Mspo	-
Best1	-
Pde1c	-
Phm	-

Figure 4.10: Network analysis of the top 100 genes upregulated in hemocytes upon *Ecc15* treatment

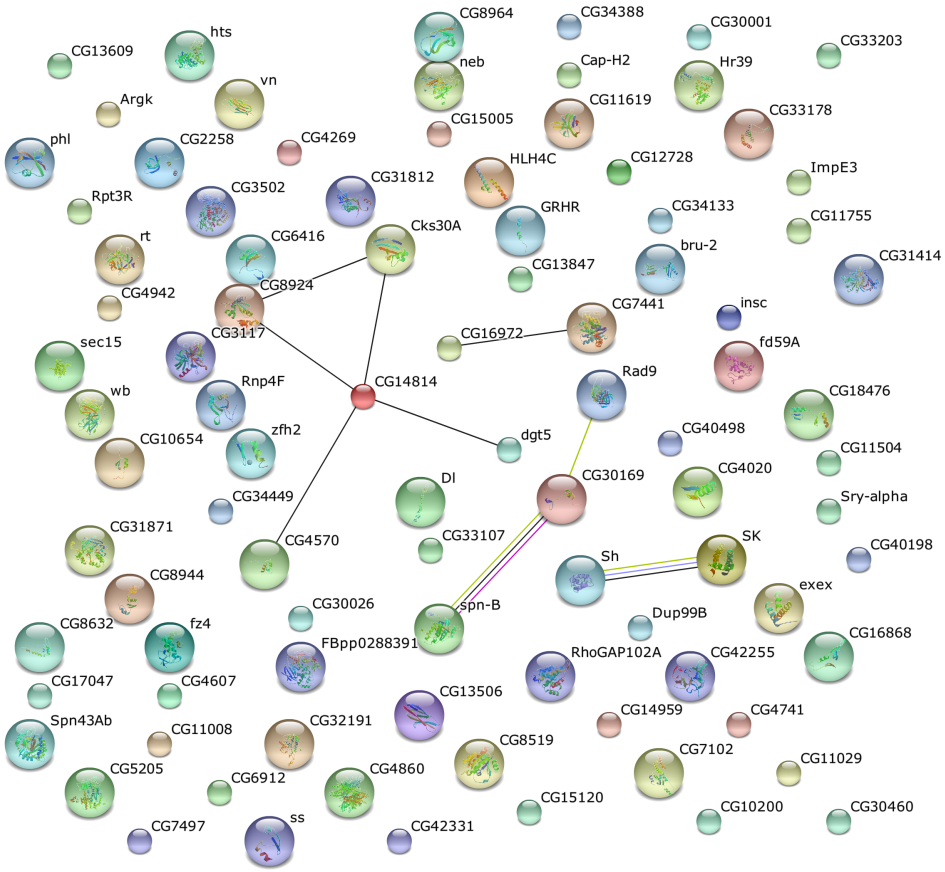
STRING network analysis demonstrated that very few interactions were predicted between genes upregulated in hemocytes by Ecc15 treatment, suggesting that expression of these genes is not increased in a large upregulatory network. Only 4 predicted interactions were noted, and only that between Phosphodiesterase 1c (*Pde1c*) and *Adenylate cyclase 3* (*Ac3*) was predicted to be a highly probable interaction. All other predicted interactions were attributed with much lower probability scores, indicating that they are less likely to have a true biological basis. Black edges reflect predicted interactions based on co-expression evidence. Blue edges reflect proposed interactions based on evidence of co-occurrence of protein products. Green edges are interactions that are predicted on text-mining evidence.



Interacting Gene A	Interacting Gene B	Probability of interaction (AU)
CG3085	*CG18371*	0.637
CG9406	*CG18371*	0.433
Ccap	*CG11475*	0.401
Pde1c	*Ac3*	0.899

Figure 4.11: Network analysis of the top 100 genes downregulated in hemocytes upon *Ecc15* treatment

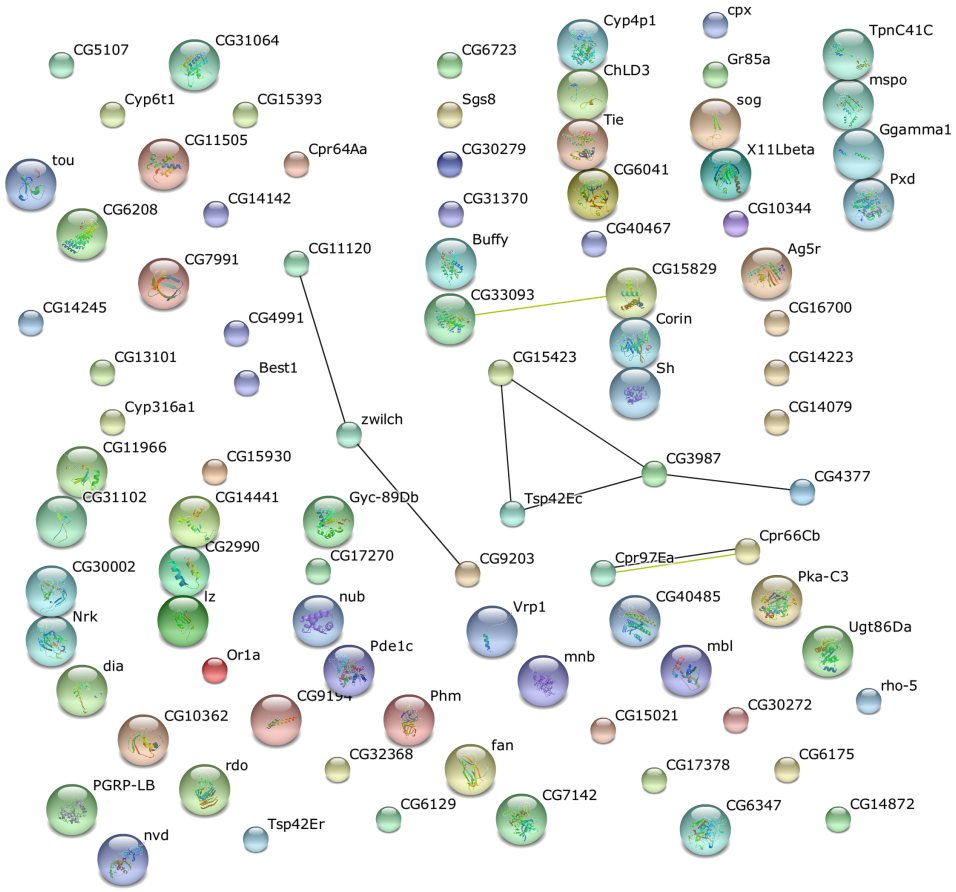
STRING network analysis detected the presence of highly simplistic downregulatory networks of genes within hemocytes that responded to *Ecc15* treatment. 4 different networks, consisting of a total of 12 nodes were identified. Annotations of genes suggested that these minor networks may function to regulate processes related to cell division, ion transport and DNA repair. However, the majority of interactions depicted were attributed low probability scores, with the exception of that between CG30169 and Rad9 (highlighted in red). Black edges reflect predicted interactions based on co-expression evidence. Pink edges represent interactions predicted on experimental evidence. Green edges are interactions that are predicted on text-mining evidence.



Interacting Gene A	Interacting Gene B	Probability of interaction (AU)
Sh	*SK*	0.530
CG30169	*Spn-B*	0.428
	Rad9	0.892
CG14814	*Dgt5*	0.565
	CG4570	0.458
	Cks30A	0.599
	CG8924	0.585
CG8924	*Cks30A*	0.402
CG16972	*CG7441*	0.585

Figure 4.12: Network analysis of the top 100 genes upregulated in hemocytes upon PBS treatment

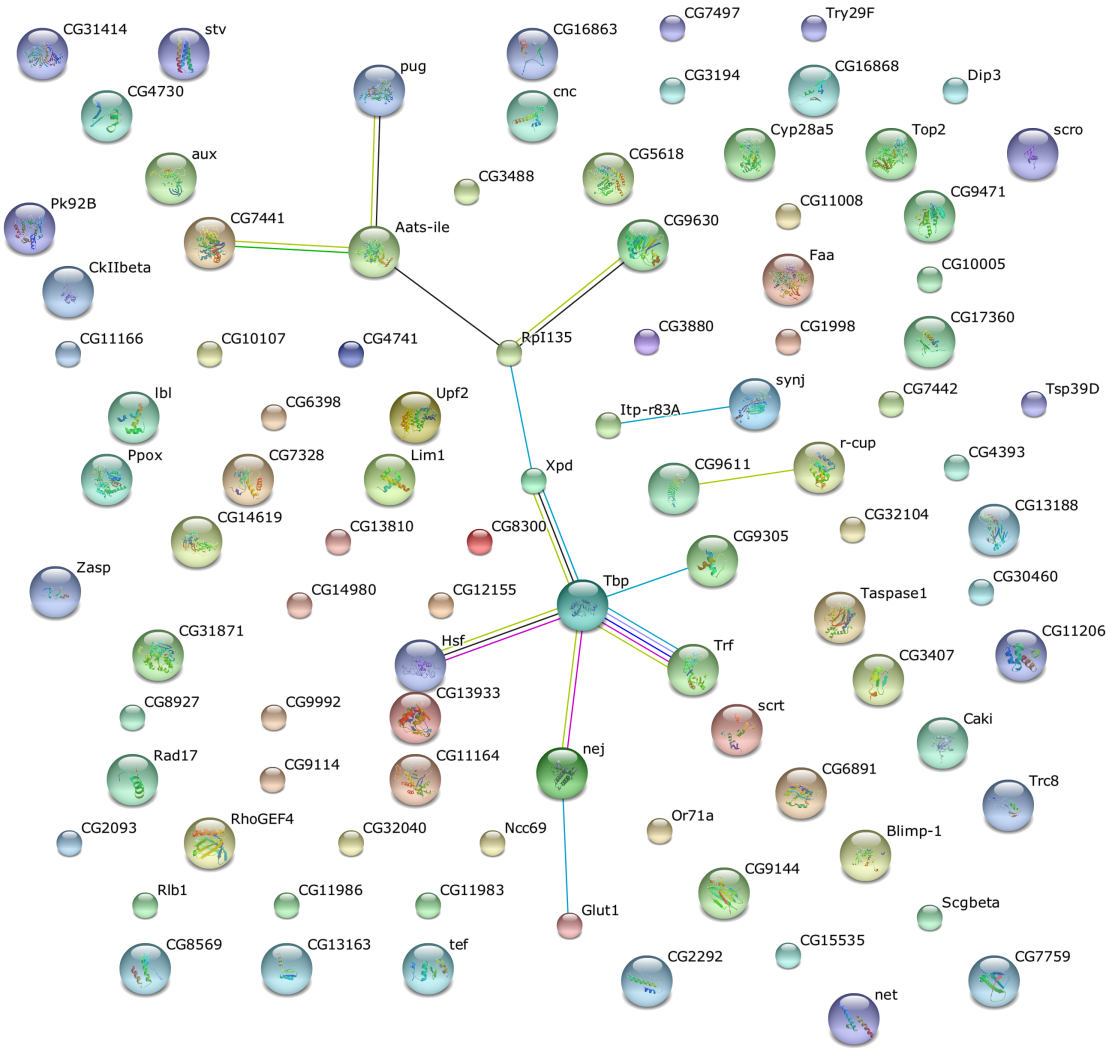
STRING network analysis of the top 100 genes upregulated in hemocytes upon PBS treatment predicted only few interactions between these genes, in three different network structures. The functions of these genes may suggest a role for these networks in the oxidation-reduction and DNA repair processes. However, none of these interactions were attributed with a high probability score. Black edges reflect predicted interactions based on co-expression evidence. Green edges are interactions that are predicted on text-mining evidence.



Interacting Gene A	Interacting Gene B	Probability of interaction (AU)
CG15423	*CG3987*	0.477
	Tsp42Ec	0.704
Tsp42Ec	*CG3987*	0.675
CG3987	*CG4377*	0.637
Cpr97Ea	*Cpr66Cb*	0.595
CG33093	*CG15829*	0.470
Zwilch	*CG11120*	0.417
	CG9203	0.413

Figure 4.13: Network analysis of the top 100 genes downregulated in hemocytes upon PBS treatment

STRING network analysis of the top 100 genes downregulated in hemocytes upon PBS treatment revealed one predicted network and two individual interactions of interest. The network identified consisted of 12 nodes and 11 edges, and was composed of genes that may have roles in the regulation of transcription. Moreover, some of these predicted interactions were associated with a probability score of ≥90% (highlighted in red), validating their existence. Black edges reflect predicted interactions based on co-expression evidence. Blue edges reflect proposed interactions based on evidence of co-occurrence of protein products. Green edges are interactions that are predicted on text-mining evidence. Pink edges represent interactions predicted on experimental evidence.



Interacting Gene A	Interacting Gene B	Probability of interaction (AU)
Tbp	*CG9305*	0.899
	Trf	0.750
	Nej	0.609
	Hsf	0.593
	Xpd	0.928
Nej	*Glut1*	0.899
Rpl135	*Xpd*	0.933
	CG9630	0.706
	Aats-ile	0.675
Aats-ile	*CG7441*	0.798

	pug	0.573
Ltp-r83A	*Synj*	0.899
CG9611	*r-cup*	0.619

4.3 Discussion

4.3.1 Transcriptional profiling confirms the immune potential of the Stage 15 *Drosophila* embryos and facilitates the identification of novel infection-responsive gene for further study

The primary aim of these experiments was to assess the nature of the global transcriptional changes upon bacterial and damage stimuli within the Stage 15 embryo, to identify potential novel infection- and damage-specific genes and ultimately to identify potential candidate genes for further study. To achieve this objective, the transcriptional profiles of Stage 15 embryos injected either with *Ecc15*, a Gram-negative bacterium, or *M. luteus*, a Gram-positive bacterial species, or sterile PBS were initially compared to that of naïve embryos; to detect if infection and damage induced modulation of immune genes within the Stage 15 embryo could be detected via the microarray methodology. Subsequently, further comparisons between PBS and bacterial injected transcriptomes permitted the identification of significantly upregulated infection genes that were either unique or common to both types of infection.

This approach confirmed that bacterial species are able to activate a range of immune responses within the Stage 15 *Drosophila* embryo, consistent with previous results chapter (Chapter 3). For instance, *Ecc15* infection induced expression of genes involved in IMD, JAK-STAT and JNK signaling, consistent with results from larval gut models (Buchon *et al.,* 2009a; Buchon *et al.,* 2009b); demonstrating the relatively advanced immune capabilities of the *Drosophila* embryo. Interestingly, *Ecc15* infection also induced the expression of *proPO-A1*, a gene involved in the melanisation cascade (Cerenius *et al.,* 2008). Whilst no melanisation had been noted within embryos upon infection (data not shown), this result would corroborate data suggesting that modulation of genes involved in the melanisation cascade occurs upon *Ecc15* infection within the embryo (Chapter 3) and thus confirm the notion that embryos are capable of mediating a melanisation response. Moreover, upregulation of genes involved in encapsulation and autophagy, as well as the immune gene *edin,* would infer that the global immune response in *Drosophila*

embryos is relatively extensive. The transcriptional profile of embryos infected with *M. luteus* further consolidated this paradigm, with the upregulation of genes involved in JAK-STAT signaling, opsonisation and phagocytosis. Moreover, the fact that genes were identified that responded uniquely to *Ecc15* or *M. luteus* infection suggests that the embryo is able to mediate complex, distinct immune responses to Gram-negative and Gram-positive infections that far surpass the basic differential expression of AMP genes.

Furthermore, one clear advantage of performing microarray screens as opposed to the study of individual candidate genes was that this approach permitted the investigation of a much wider range of genes in concert, and hence identified novel and interesting effects of infection upon the host transcriptome. One such interesting result was the upregulation of *PGRP-LF* specifically by *Ecc15* infection. *PGRP-LF* is a negative regulator of the IMD signaling cascade (Persson *et al.,* 2007; Maillet *et al.,* 2008; Basbous *et al.,* 2011); its mechanism of action remains unclear, although evidence suggests that it may either act as a decoy receptor for PGN (Persson *et al.,* 2007) or compete with PGRP-LC within multimer complexes to bind PGN, hence dampening down IMD pathway activation (Basbous *et al.,* 2011). As such, it was relatively surprising that *PGRP-LF* expression was so highly upregulated by *Ecc15* infection, especially as induction of Gram-negative AMP genes was also detected. Since *M. luteus* infection did not have the same effect on *PGRP-LF* induction, the increase observed must be either Gram-negative or *Ecc15* specific. Kleino (2010) demonstrated that S2 cells incubated with *E. coli* also induced *PGRP-LF* expression at 4 hours after application. Thus, one possible explanation may be that the embryonic host specifically upregulates expression of *PGRP-LF* to ensure that the acute immune activation required to eliminate the pathogen does not lead to a chronically upregulated immune state, which could be potentially damaging. The fact that the upregulation within the embryo is observed at an earlier time-point post infection (2 hours) is potentially a testament to the highly immunogenic nature of *Ecc15* itself (Basset *et al.,* 2000; Tzou *et al.,* 2000; Foley and O'Farrell, 2004), thus necessitating a less prolonged immune response. Alternatively, this early upregulation could indicate subversion of the embryo host immune machinery

by *Ecc15*; to dampen down the immune response so that the infection may continue to thrive. This paradigm could be similarly applied to the upregulation of *Bruce*, a negative regulator of autophagy (Hou *et al.,* 2008; Nezis *et al.,* 2010). The observed upregulation of *Bruce* expression upon *Ecc15* infection may suggest an inhibition of the autophagic clearance of invading bacteria, which is not necessarily concordant with the requirements of the embryonic host. However, it is well established that overstimulation of the autophagy machinery can subsequently lead to cell death (White, 2008), which also may not be favourable for the embryo given the potentially wide and random spatial distribution of concentrated bacteria within its tissues. Thus, to determine if upregulation of gene candidates such as *PGRP-LF* and *Bruce* are a result of host subversion by *Ecc15* or the host's response to prevent a run-away immune response, further experimental evidence is required. One way to address this issue would be to monitor *PGRP-LF* or *Bruce* expression in the embryo upon infection with a similarly pathogenic Gram-negative bacterium, to determine if the changes in *PGRP-LF* and *Bruce* are specific to *Ecc15*.

Furthermore, analysis of the embryo transcriptome upon *M. luteus* infection also yielded novel, interesting results. The observation that *TepI* expression was induced uniquely upon *M. luteus* infection was of notable interest, as relatively little is known about the function of this tep family member in *Drosophila*. The nature of this embryonic expression would suggest that *TepI* is beneficial to the embryo host in mediating an immune response to Gram-positive infection, and dispensable for the response to Gram-negative infection. However, this is in contradiction to previous work by Stroschein-Stevenson *et al.* (2005), who found no positive or negative benefits of *TepI* expression on the phagocytosis of *E. coli*, *S. aureus* or *C. albicans* in S2 cells. However, it could be argued these results may not be entirely indicative of TepI activity or its role *in vivo*. Moreover, given the fact that producing immune effectors is a costly activity for the host (Sadd and Siva-Jothy, 2006; Sorci and Faivre, 2009), it would be surprising if the observed upregulation in *TepI* expression did not confer some form of benefit for the embryo upon infection. Therefore, further investigation would be required to validate this result and to

establish if *TepI* expression is restricted to the embryonic response to Gram-positive pathogens.

Interestingly, despite the relatively disparate effects of *Ecc15* and *M. luteus* infection on the embryonic transcriptional profile discussed so far, both infection types also induced the upregulation of a common subset of genes. These genes formed a large proportion of the 200 most significantly upregulated genes for each infection type (36.5%). This would broadly suggest that a large proportion of the global response to infection is relatively aspecific in terms of infection-type. This notion is confirmed by the observation that the functions of many of these commonly upregulated genes are not immune-specific, but nevertheless may suggest a more general response to stress or an increase in normal cell operations, possibly to cope with the demands of a sudden and urgent global transcriptional change. This perspective is confirmed to an extent by the results of De Gregorio *et al.* (2001), whose examination of the transcriptional profile of adult flies after infection with a mixture of *M. luteus* and *E. coli* also documented modulation of genes with more general functions, such as induction of genes encoding lipases and lysosomal enzymes. Nevertheless, the majority of remaining gene hits reported by De Gregorio *et al.* (2001) had at least a putative if not clear immune annotation. This is in contrast to the results of our microarray study, as the only gene with a clear immune function that was commonly upregulated by both *Ecc15* and *M. luteus* infections was *spz4. Spz4* is a homologue of *Spz* that was discovered by Parker *et al.* (2001) via iterative searching of the *Drosophila* genome. Due to its sequence similarity to *Spz*, *Spz4* has thus been inferred to be capable of activating Toll signaling (Parker *et al.* 2001). Expression studies highlighted that *Spz4* is strongly expressed in larvae and adults, and has been suggested to play an ancillary role in the response to fungal or Gram-positive pathogens (Parker *et al.,* 2001). Therefore, the fact that *Spz4* expression was upregulated upon both *Ecc15* and *M. luteus* infection within the embryo is surprising and it is interesting to speculate about the particular role that *Spz4* may be playing in these distinct responses. Similar to the hypothesis of Parker *et al.* (2001), *Spz4* could potentially be providing supporting role in the embryo response to *M. luteus*; amplifying the

amount of ligand available for Toll pathway activation. A possible function for *Spz4* upon *Ecc15* infection may involve the cross-activation of the IMD and Toll pathways. There is much evidence to suggest that cross-talk exists between the IMD and Toll signaling cascade (Bangham *et al.,* 2006; Matskevich *et al.* 2010), but any mechanistic detail describing how this might work in practice remains relatively elusive. Thus, the expression of *Spz4* upon *Ecc15* infection may contribute to this phenomenon. Moreover, this proposed role for *Spz4* would potentially explain the high levels of *Def* expression consistently noted upon *Ecc15* infection, both in the embryo system and in other *Drosophila* immunity models (Lemaitre *et al.,* 1997; Buchon *et al.,* 2009). To determine the contribution of *Spz4* to the total embryo immune response, infection studies could be performed with *Spz4*-deficient embryos, permitting the observation of the viability of these embryos or the AMP gene induction post infection.

In a broader context, it is also interesting to speculate if infection by Gram-positive and Gram-negative bacterial species leads to significantly different transcriptomic profiles. Clearly, a common subset of genes is modulated upon both *Ecc15* and *M. luteus* infection, indicating that some genes may simply respond in a manner that is simply ‘infection’ or ‘non-infection’. However, the fact that further different subsets of genes are modulated upon *Ecc15* or *M. luteus* infection alone is suggestive that Gram-negative and Gram-positive bacterial species may induce their own individual transcriptomic ‘signature’ within the host. It is not possible to confirm this notion from the data presented; transcriptomic studies could be performed on a wider range of Gram-negative and Gram-positive bacterial species, such as *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus* and *Enterococcus cloceae*, to determine if there are genes that are modulated solely in response to Gram-negative and Gram-positive infections. It would also permit the identification or confirmation of aspecific genes that are modulated upon infection. It may also be interesting to correlate differences in host transcriptomic signatures and the degree of relatedness between infecting bacteria species; for instance, do more closely related species produce more significantly similar transcriptional profiles than

more distantly related species, and if so are there marker genes to indicate infection by a particular genera of bacteria?

Therefore, these results would suggest that the embryonic global response to bacterial infection is relatively comparable to that of other *Drosophila* immunity models, further substantiating the status of the *Drosophila* embryo as a valid immunity model. Moreover, this data highlights the very distinct nature of the embryonic response to Gram-negative and Gram-positive infections that is more complex and extensive than simply a differential expression of AMP genes, leading to the identification of potential candidate genes that may be considered for validation by RT-qPCR and further study. In particular, it is interesting to speculate of the existence of Gram-positive and Gram-negative global transcriptomic signatures, which could be discerned by conducting further genome-wide transcriptomic studies on a larger range of bacterial species.

4.3.2 Sterile laser ablation upregulates AMP genes as well as a network of cuticular proteins genes

Further microarray studies permitted the comparison of the transcriptional profile of Stage 15 embryos wounded by sterile laser ablation to that of non-wounded embryos; to further examine the range of genes modulated upon damage within the Stage 15 embryo and to potentially identify novel wound-responsive genes. Analysis of the 100 genes demonstrating the most significant modulation upon laser ablation revealed significant upregulation of genes involved in cuticle development and structure, as well as mRNA splicing. Further GO analysis of these genes demonstrated confirmed an enrichment of GO terms relating to cuticular functions and extracellular space components, highlighting the importance of cuticle development and integrity upon wounding. Of particular note was that many of the cuticular genes upregulated upon wounding within the embryo appeared to be interacting in an upregulatory network, incorporating many genes of unknown function and hence permitting the inference of provisional functional annotations for these

genes. The cuticle, a structure composed of chitin fibrils embedded in a protein-lipid matrix (Cohen, 1987), is essential for maintaining the structural integrity of *Drosophila* larvae (Ostrowski *et al.,* 2002), as well as serving as a defense barrier against infection by pathogens (Tzou *et al.,* 2002). Its importance is further highlighted by the embryonic lethality observed when any one gene required for proper cuticle deposition or morphogenesis is zygotically disrupted (Ostrowski *et al.,* 2002). Interestingly, epidermal cells deposit the cuticle during Stage 16 of embryogenesis (Ostrowski *et al.,* 2002); at a time-point close to the time of wounding by laser ablation at Stage 15. Thus, with a significant structural failure of the epidermis, it could be expected that genes related to cuticle development might be downregulated as a direct result. However, we know that the opposite is true; that genes belonging to the Twdl, Cpr and Lcp families that regulate cuticle development are upregulated upon laser ablation within the Stage15 embryo. Therefore, it might be speculated that expression of genes such as Twdl family members, whose protein products are inserted into the cuticular matrix (Guan *et al.,* 2006) and thus contribute directly to cuticle deposition, is upregulated in the remaining epidermal cells, to compensate for the loss of a significant number of cells that would have contributed to cuticle deposition and ultimately to ensure that embryo viability is preserved. A further reason for the upregulation of a cuticular gene network may be to preserve the structure of the embryo and protect against infection from opportunistic pathogens whilst epidermal damage is repaired.

Analysis of the wounded embryo transcriptome also provided further confirmation that damage alone can stimulate AMP gene induction, and hence an immune response, within the *Drosophila* embryo. In light of the discovery that immune gene induction upon infection is greater when accompanied by injury (Braun *et al.,* 1998) and that increased survival rates upon bacterial infection are noted in flies that have received a previous injury (Apidianakis *et al.,* 2005), it could be speculated that induction of AMP genes by injury may play a role in priming the host defense to future infection or structural compromise of the vitelline membrane which in turn would permit the entrance of pathogens into embryo tissue. AMP genes that were significantly induced

included members of the *Att, Cec, Drs* and *Mtk* AMP classes. This is in concordance with the work by Stramer *et al.* (2008), who demonstrated that sterile laser ablation could induce the expression of a *Drs-GFP* reporter in fat body and hemocytes in *Drosophila* embryos of a similar stage. Moreover, the range of AMPs that were significantly upregulated by sterile laser ablation was relatively consistent with that observed by Patterson *et al.* (2013) upon pinch wounding of *Drosophila* embryos. However, the fold changes in expression of AMPs upon pinch wounding noted by Patterson *et al.* (2013) was generally much greater than those observed in our sterile ablation system. For instance, 3.89-fold change in *AttA* expression was observed upon sterile ablation as opposed to the 11.6-fold change observed by Patterson *et al.* (2013). This could be attributed to the nature of damage inflicted; whilst sterile laser ablation in embryos removes only one epithelial population in a concentrated area, mechanical damage caused by puncturing may lead to much greater disruption of multiple layers of tissue, suggesting that AMP production in response to damage is proportionate to the level of damage inflicted. Regardless of the levels of induction, it is clear that damage induces a preferential induction of a subset of AMPs. For example, of the AMP genes significantly upregulated by sterile laser ablation, *AttA, AttD* and *Dro5* were particularly highly induced. This combination encompasses both Gram-negative and Gram-positive AMPs, suggesting that there is no bias towards the expression of a particular broad AMP class. Further experiments using *Drosophila* transgenic AMP reporter lines would enable the determination of the site of expression for these AMP genes upon sterile laser ablation within the Stage 15 embryo, and potentially give insight into their individual roles upon damage.

However, the range of immune genes upregulated by sterile laser ablation did not encompass only those encoding AMPs. Another interesting observation was that *PGRP-LD* expression was significantly upregulated within wounded embryos. This is in contrast to the work of Patterson *et al.* (2013), whose authors did not observed any significant increase in *PGRP-LD* expression upon pinch wounding of *Drosophila* larvae, despite the increased expression of many other PGRP genes, including *PGRP-LA, LB, LC, LF* and *SA*.

Moreover, no significant upregulation of *PGRP-LD* was noted by Stramer *et al.* (2008) upon sterile laser ablation of *Drosophila* embryos. Relatively little is known about the *PGRP-LD* gene itself; Werner *et al.* (2000) revealed that the 5' region of the *PGRP-LD* transcript contains an ORF which is related to the human *PMI* gene. *PMI* plays a role in the regulation of mitochondrial morphogenesis (Rival *et al.,* 2011), thus suggesting that *PGRP-LD* may have a similar role in meeting cellular energy demands, as opposed or in addition to being a pattern recognition receptor. However, Werner *et al.* (2000) also noted that *PGRP-LD* expression was greatly enriched in hemocytes, and combined with the observation that PGRP-LD is able to bind PGN (Goto and Kurata, 2006), this further suggests that *PGRP-LD* has some immune function. Since PGRP-LD does not play a role in phagocytosis (Ramet *et al.,* 2002), and given its ability to bind PGN, it could be speculated that PGRP-LD regulates the systemic response in some manner. Therefore, the precise role that *PGRP-LD* may be playing upon sterile laser ablation within the Stage 15 embryo is highly speculative, but may couple together the regulation of AMP production upon wounding with the energy demands of the immune response. The concept of cross-regulation of innate immunity and the energy requirements of cells is not novel; Becker *et al.* (2010) demonstrated that metabolism and innate immunity could be co-regulated by FOXO in response to the fluctuating energy status of cells and tissues, independent of pathogen stimuli. Moreover, Karpac *et al.* (2011) elucidated a relationship between the AMP response to DNA damage and both IMD and insulin/IGF signaling, potentially coordinating growth and metabolic activities across tissues. Therefore, the notion that *PGRP-LD* may represent part of a similar damage response to sterile laser ablation may be well supported. However, due to overlap of the 3' untranslated region of *PGRP-LD* with an ORF on the opposite DNA strand (Werner *et al.,* 2000) and thus the potential for transcription of multiple mRNA species from the same region, careful further validation of this potential candidate would be required.

Aside from immune and cuticle related genes, a collection of U5 snRNAs were also upregulated in Stage 15 embryos upon sterile laser ablation, inferring an upregulation in mRNA splicing. One possible explanation could reside in signal-dependent alternate splicing mechanisms, whereby damage signals

can result in the increased inclusion of alternate exons; for instance, DNA damage to S2 cells has been demonstrated to cause the increased inclusion of *TAF1* alternate exons (Marengo and Wasserman, 2008). This process relies on the stability of splice site complexes, as binding of splice complexes to alterative splice sites is often weaker than to constitutive sites (Marengo and Wasserman, 2008). U5 snRNAs, such as those upregulated in the embryo upon laser ablation, have previously been suggested to play a role in the stabilization of exon alignment (Teigelkamp *et al.,* 1995), and thus it may be speculated that upregulation of U5 snRNAs by the embryo upon laser ablation may permit the increased stabilization of the spliceosome complex at alternative exons; shifting the embryonic transcriptional response upon damage stimuli. An alternative explanation may take into account the enormous transcriptional response within the embryo upon damage stimuli. Previous studies have elucidated that hundreds of genes are significantly modulated upon wounding or the infliction of damage; 240 genes were significantly modulated in larvae damaged by ionizing radiation (van Bergeijk *et al.,* 2012). This no exception within the embryo, as 933 genes demonstrated significant modulation upon sterile laser ablation. Thus, the upregulation of snRNAs may in fact reflect the increased amount of mRNA processing that must be carried out by the embryo in order to launch an effective damage response. This would therefore imply that processes which provide support and maintenance to the damage response and processes that mediate its direct effects are of equal importance within the embryo.

Therefore, transcriptional profile analysis of laser-ablated embryos revealed that a potential balance between the damage-induced immune response and other biological processes, such as those required for correct development, maintenance of the transcriptional output and energy homeostasis. Moreover, this screening has permitted the identification of range of genes that may play as of yet undefined roles in the embryonic response to damage. The expression levels of these candidates now require further validation by an independent methodology, such as RT-qPCR, to determine if they ultimately have any true biological basis. Furthermore, the large proportion of wound-induced genes that currently have no biological function attributed also

represents a substantial list of novel candidate genes, whose expression upon sterile laser ablation could firstly be validated by RT-qPCR protocols. Subsequently, experiments could be designed to gain a greater understanding of their function; for example, knocking-out or overexpressing these genes within both non-wounded and wounded embryos would potentially enable the elucidation of their potential biological function.

4.3.3 Transcriptional profiling provides novel insight into the role of the hemocyte upon infection and damage

A parallel aim of this analysis was to more specifically assess the contribution of embryonic hemocytes towards the embryonic immune response, by examining the transcriptional profile of this immune cell type upon injection with *Ecc15* or PBS; to assess the immune role of the hemocyte upon infection and damage stimuli respectively. One interesting feature of the resulting hemocyte transcriptional profiling data was that genes involved in the oxidation-reduction processes were induced upon both PBS and *Ecc15* treatment. This included genes encoding various CYPs, ninaG, peroxidase and laccase2. This is in contrast to results by Johansson *et al.* (2005), who noted a decrease in expression of Cyp genes in mbn-2 cells upon *E. coli* infection. One potential explanation for this phenomenon may involve ROS production. *Ecc15* is known to induce the activation of Duox in *Drosophila* gut epithelia and hence H_2O_2 production, via the release of bacterial-derived uracil (Lee *et al.,* 2013). Moreover, Lee *et al.* (2013) demonstrated that an acute ROS response controlled via this mechanism was required for efficient elimination of bacteria, whereas chronic activation of this system was detrimental to host survival to infection. Thus, the observed increase in expression of genes encoding proteins with oxidoreductase activity in hemocytes upon *Ecc15* infection may potentially contribute to this antibacterial response, generating further ROS, whilst upregulation of genes such as peroxidase may prevent overexposure of the host tissues to ROS. It is known that hemocytes are responsive to ROS signals, such as the hydrogen peroxide released from epithelial wounds (Moreira *et al.,* 2010), thus it may not be

surprising that they may have a direct transcriptional response to this genre of stimuli. Similarly, previous work has demonstrated that wounds release H_20_2 as a damage cue to recruit hemocytes to wounds (Moreira *et al.,* 2010); therefore, upregulation of oxidoreductase genes upon the damage stimuli resulting from PBS injection may potentially further amplify this damage response via the production of ROS from hemocytes themselves, initiating further hemocyte recruitment to damage sites. Moreover, this effect on oxidoreducctase gene expression may also be speculatively coupled to an increase in expression of genes involved in DNA repair, such as *Mei-W8*; permitting the hemocyte to protect itself against the well-documented detrimental effects of ROS on DNA integrity (Henmani and Parihar, 1998; Cooke *et al.,* 2003). Interestingly, the Cyp genes upregulated in hemocytes upon *Ecc15* infection have also previously been associated with heme and iron binding activity (Flybase Curators, 2004). Based on the observation that bacteria require iron for effective growth (Guerinot, 1994), this may represent another form of antibacterial response; Cyps may sequester heme or iron to limit the access of *Ecc15* bacteria to this crucial resource. The fact that these genes are upregulated within hemocytes also corroborates this theory, as the Cyp genes in question would potentially be expressed in the ideal location for sequestering iron and heme. However, this effect may not be hemocyte-specific, as the transcriptional profiles of the total embryo response also included the upregulation of oxidoreductase encoding genes, some with predicted iron binding properties. In this sense, the hemocyte and total transcriptomes share a common feature and hence it may be that hemocytes play a distinct role within a global embryonic oxidation-reduction response.

Moreover, the hemocyte transcriptome upon *Ecc15* infection is similar to that of the total embryos in another regard; genes involved in more general processes, such as mRNA processing and proteolysis, are also upregulated in hemocytes upon infection with *Ecc15*. Genes involved in proteolysis have previously been observed to be upregulated upon microbial challenge of S2 and mbn-2 cells (Johansson *et al.,* 2005), confirming their importance in the hemocyte immune response. Thus, genes encoding endopeptidases, such as *pcl, tld* and *Nep5*, may have putative functions in the degradation of bacterial

components upon phagocytosis. Similar to the reasoning for the increased expression of genes involved in mRNA splicing, the upregulation of mRNA processing genes may occur to counteract the increased demands placed on transcriptional processes within the hemocyte; to efficiently upregulate expression of effectors required for the cellular immune response. However, this is somewhat at odds with the observation that cells shut down overall protein synthesis in response to oxidative stress (Khong and Jan, 2010); a situation which hemocytes must clearly be experiencing considering their extensive expression of genes involved in oxidation-reduction. Therefore, the fact that hemocytes still potentially increase their transcriptional efforts to respond to infection stimuli under oxidative stress conditions suggests that an immune response is prioritized over a stress response in hemocytes. Previous work has documented the ability of hemocytes to prioritise competing signals (Moreira *et al.,* 2010), thus this result may represent another link within the hemocyte prioritization chain.

However, other results would potentially downplay the role of hemocytes in the embryonic immune response. For instance, expression of *insc*, a gene previously characterized to be necessary for survival to *V. cholorae* infection (Berkey *et al.,* 2009), was downregulated in hemocytes upon *Ecc15* infection. Expression of *Hr39*, a target of 20-HE signaling and with a role in autophagic cell death (Takemoto *et al.,* 2007), was also downregulated in hemocytes upon *Ecc15* infection. Therefore, these changes may infer the relative dispensability of hemocytes, as well as the responses of these genes, within the embryonic immune response. However, from the perspective of the host, these observations would not accord with survival to *Ecc15* infection. Previous chapters have cited the potential of *Ecc15* to subvert the 20-HE signaling machinery of the embryonic host, to potentially switch off the host immune response and allow the infection to grow unchecked (Chapter 3). These results may suggest a similar phenomenon, especially given that *Hr39* is a target of 20-HE signaling (Horner and Thummel, 1995), indicating a direct interaction between *Ecc15* and the hemocyte transcriptional response. In addition, other downregulated genes included *wb* and *phl*, which have been implicated in tracheal development (Martin *et al.,* 1999; Jiang and Edgar,

2009). Given the importance of the trachea in mediating survival to infection via 20-HE signaling and reducing bacterial load, these changes may also represent an attempt by *Ecc15* to inhibit host immunity. A simple manner in which this paradigm may be tested would be to conduct mortality studies of embryos with deficiencies in these candidate genes post infection with *Ecc15*; to determine if their survival to infection is significantly different to that of WT embryos.

In terms of the hemocyte transcriptional response to PBS injection damage, the proposed functions of the subsequently modulated genes determined via GO analysis were relatively consistent with those of genes modulated upon *Ecc15* infection in hemocytes; indeed a modest percentage of genes upregulated or downregulated were common to both treatments, suggesting that damage via injection process has a significant impact on the hemocyte transcriptional response. Thus to identify hemocyte-specific infection genes in future experiments, it will be important to account for this contribution and to compare transcriptional profiles of PBS and *Ecc15* treated hemocytes directly to compensate for this. However, some interesting features of the PBS treated hemocyte transcriptome can still be observed. For instance, it is interesting that expression of genes involved in 20-HE synthesis, such as *nvd* and *phm* was upregulated upon damage induced via PBS injection. This is in contrast to work by Hackney *et al.* (2012), who observed that tissue damage disrupted 20-HE biosynthesis in first instar larvae but did not propose any theory as to why a response of this nature may be occurring. Thus, the differences observed between the work of Hackney *et al.* (2012) and results from embryonic hemocytes may infer a hemocyte-specific activation of 20-HE signaling. As 20-HE signaling has been demonstrated to play a role in activating innate immune-competence within the embryo (Chapter 3), it is interesting to speculate that this result may reflect a mechanism by which hemocytes are able to produce a local 20-HE response to activate innate immune mechanisms.

Taken together, these preliminary results suggest that the hemocyte transcriptomes upon infection and damage share characteristics with those of

the total embryo, such as the upregulation of genes involved in oxidation-reduction and mRNA processing. However, there is also evidence to suggest that hemocytes may play a more distinct role in the immune responses to infection and damage, via the proposed direct interactions between *Ecc15* and the hemocyte transcriptional response, as well as the potential propagation of 20-HE signaling upon damage stimuli. However, these results and subsequent discussion remain highly speculative since only one replicate of the hemocyte microarray experiment has been performed. Addition of data from a further two replicates will allow the identification of genes which are significantly modulated within hemocytes upon *Ecc15* infection and damage induced by PBS injection. As a result, networking analysis may result in a more coherent network of up- or down-regulated genes. Moreover, the comparison of hemocyte and total embryo transcriptional profiles would permit the identification of hemocyte-specific damage and infection genes.

4.4 Conclusions

To conclude, analysis of the transcriptional response of the *Drosophila* embryo to infection confirmed the immune potential of this system; that embryos at Stage 15 of development are able to mediate a diverse immune response, indicative of the activation of many signaling cascades involved in the systemic and cellular immune response. Furthermore, wounding via sterile laser ablation was also able to mediate a distinct transcriptional response within the embryo, including the significant induction of a subset of AMP genes and the expression of a network of cuticular genes, as well as genes involved in mRNA splicing. Analysis of the hemocyte transcription upon infection and damage elucidated that these immune cells may play a role regulation of the immune response via 20-HE signaling and production of ROS, although this remains speculative and subject to validation. As such, analysis of these transcriptional profiles has been successful in identifying candidate genes for further validation and study.

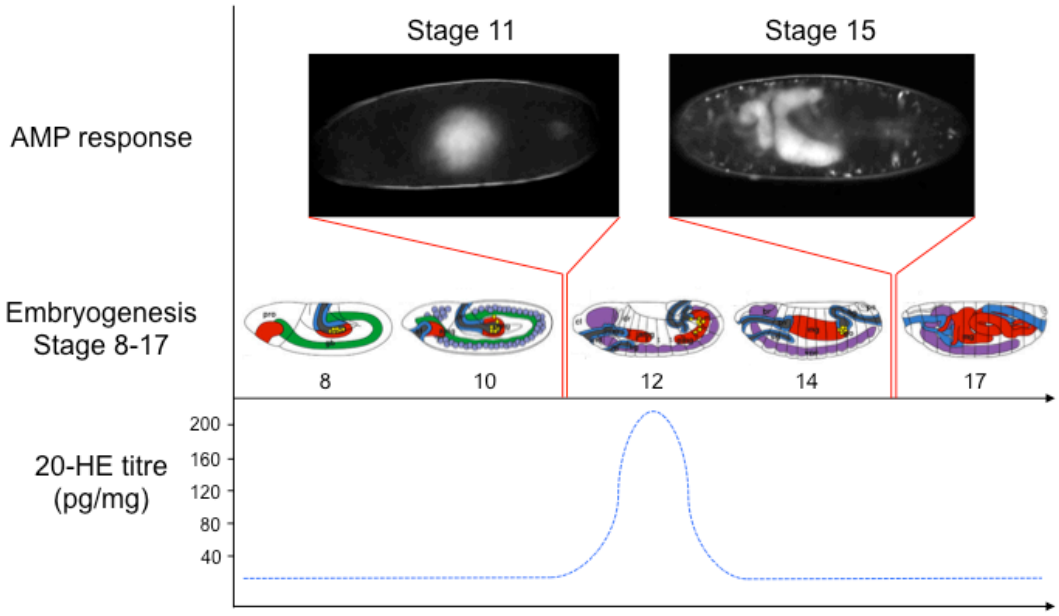
Chapter 5: Final Discussion

The central aim of this project was to develop a more detailed and intricate model of bacterial infection using the *Drosophila* embryo, and to assess its potential as a viable immunity model. A further key aim of this project was to determine at what stage of development *Drosophila* embryos begin to demonstrate immune competence and what biochemical signaling pathways may drive this immunological phenomenon. Having established the immune capacity of the embryo in terms of its ability to respond to bacterial challenge, subsequent objectives focused on the determination of global and hemocyte-specific transcriptional changes within the embryonic system upon infection with Gram-negative or Gram-positive microbes, or sterile damage induced by either laser ablation or sterile injection procedures. This would enable the identification of the novel genes that were modulated in *Drosophila* upon infection or damage.

Initial results demonstrated that *Drosophila* embryos were able to mount an effective AMP response to a range of bacterial infections, and that this was mediated mostly via the canonical *Drosophila* systemic immunity pathways. Moreover, multiple facets of the cellular and systemic response were observed to be functional within the embryo system, operating in a manner that was concordant with the host defense mechanisms of the adult fly and larval models. Subsequently, it was demonstrated that 20-HE signaling was crucial for the maturation of the embryonic immune system, with the 20-HE pulse during mid-embryogenesis proposed to switch on embryonic immune competence, as detailed in Figure 5.1. Results also implied that bacterial pathogens, such as *Ecc15*, might be able to subvert the embryonic 20-HE signaling machinery to prevent their elimination by host defense mechanisms. Transcriptional profiling studies identified a range of potential candidate genes for further study, such as *Ecc15*- and *M. luteus*-unique and common genes, genes potentially acting as targets of host immune system subversion for *Ecc15*, and a cuticular gene network which was upregulated upon wounding via sterile laser ablation.

Figure 5.1: Proposed Model of the Origin of Immune Competence within the *Drosophila* Embryo.

Infection studies carried out within this project enabled the development of a model to potentially explain the differences observed in the immune competence of early and late stage *Drosophila* embryos. Embryos at relatively early stages of development were demonstrated to be relatively immune incompetent, being unable to mount an *Drc-GFP* response to bacterial infection, resulting in high rates of embryo mortality. This was in stark contrast to the response of later Stage 15 embryos, which exhibited a highly robust AMP response, as well as relatively diverse overall immune response, to bacterial species, with subsequently high survival rates to infection. Considering the timing of the 20-HE pulse at mid-embryogenesis, as well as results which demonstrated that Stage 11 immunity could be rescued via treatment with 20-HE, it would infer that the 20-HE pulse may be responsible for the maturation of the embryonic immune response. Images of embryogenesis were taken from Hartenstein (1993). 20-HE titre values were based on values from Thummel (2001).


Stage 11
Stage 15
AMP response
Embryogenesis
Stage 8-17
8
10
12
14
17
20-HE titre
(pg/mg)
200
160
120
80
40

5.1 AMP expression does not confer any survival benefit upon *Ecc15* infection

A large proportion of this project was dedicated to studying the ability of the *Drosophila* embryo to express AMP genes upon bacterial infection, as a read-out of the embryonic immune potential. However, the resulting data may also reveal a novel perspective on the requirement of AMPs, suggesting that AMP bacterial killing may not be the most crucial factor in a successful *Drosophila* immune response. For instance, whilst the aforementioned interactions of *Ecc15* with the host 20-HE signaling machinery may in part have explained the moderate mortality associated with this specific bacterial infection in Stage 15 embryos, it did not fully indicate why Stage 11 embryos continued to exhibit high mortality upon co-administration of 20-HE with *Ecc15*. Upon co-administration of *Ecc15*+20HE, no significant improvement to Stage 11 embryo survival was noted compared to control embryos receiving only *Ecc15* treatment. In fact, Stage 11 survival remained highly significantly different to Stage 15 survival upon *Ecc15*+20-HE treatment despite the rescue of AMP expression within these embryos, suggesting that even by bypassing the detrimental effects of *Ecc15* on 20-HE synthesis and signaling effectors, decreasing bacterial load and compensating for the immune naivety of Stage 11 embryos, that this was not sufficient to rescue the Stage 11 embryo viability. Thus, these findings indicate that AMP production does not render the *Drosophila* embryo infallible to infection; in fact, it would indicate that AMPs do not confer any intrinsic survival benefit to the Stage 15 embryo upon *Ecc15* infection, and thus do not protect against at least infection by this bacterial species. In this sense, this concept is contradictory to previous work carried out in the *Drosophila* model. For example, work by Tzou *et al.* (2002) demonstrated that the genetic overexpression of *Def* via the Gal4-UAS system in *imd/spz* double mutant flies effectively removed the deleterious effects of *S. aureus* or *B. subtilis* infection on fly survival, thus demonstrating that overexpression of a single AMP in vivo can reverse the effects of immunodeficiency. Furthermore, the requirements of Toll and IMD signaling in the response to Gram-positive and Gram-negative bacterial infections respectively (Lemaitre *et al.,* 1996; Hoffmann and Reichart, 2002; Rutschmann *et al.,* 2002; Leulier *et al.,* 2006) would hint at the indispensable contribution of

AMPs to the total host response in resistance to bacterial infection. However, there is evidence to equally support the idea that AMPs do not confer ultimate protection against bacterial infection. For instance, this effect has also been observed in mammalian systems; mice treated prophylactically with an inducible AMP candidate extracted from *Sarcophaga perigrina* did not exhibit any significantly improved survival upon infection with *S. aureus* or *E. faecalis,* despite the extracted AMP demonstrating high antibacterial activity *in vitro* (Nakajima *et al.,* 1997; Okuyama-Nishida *et al.,* 2009). Therefore, whilst AMP induction is doubtless a cornerstone of an effective *Drosophila* immune response, the degree to which they ultimately protect the host organism and contribute to long-term survival remains somewhat conflicted.

Despite the contradictory nature and setting of the data within the current and potentially complex paradigm, the question remains as to why Stage 11 embryo viability is not increased upon 20-HE treatment, despite an apparent rescue of AMP expression. It could be argued that treatment with 20-HE, a potentially potent immune activator (Flatt *et al.,* 2007; Rus *et al.,* 2013), could induce chronic activation of the Stage 11 immune system and lead to eventual embryo mortality, given the evidence that chronic or inappropriate activation of NFκB-mediated immune responses in both *Drosophila* and mammalian systems can result in a chronic inflammatory state and reduced lifespan (DeVeale *et al.,* 2004; Haefner, 2006; Libert *et al.,* 2006; Nylaende *et al.,* 2006; Ram *et al.,* 2006). However, since Stage 11 embryos treated with 20-HE alone did not exhibit any significantly increased mortality, differential development or any evident expression of *Drc-GFP*, this explanation seems unlikely. This notion is supported by the fact that heat-inactivation of *Ecc15* does not lead to a significant decrease in Stage 11 embryo viability, suggesting that *Ecc15*-associated mortality is a result of interaction between the host and live pathogen, as opposed to a passive overstimulation of the systemic immune response.

A further possibility may be that whilst 20-HE treatment was able to enhance AMP production in Stage 11 embryos to significantly decrease the bacterial burden, it was not able to overcome all effects of *Ecc15* infection, with the

result that Stage 11 viability was not rescued. From infection studies in Drosophila larvae, *Ecc15* was shown to cause drastic remodeling of the gut; including a dramatic loss of cell numbers and significant decrease in gut width (Buchon *et al.,* 2010), to which the developing embryonic gut may prove more susceptible than its adult or larval counterparts. Moreover, *Ecc15* infection has also been demonstrated to induce the expression of enzymes responsible for the detoxification of ROS in gut epithelia (Buchon *et al.,* 2009a), suggesting that *Ecc15* infection prompts a significant host epithelial ROS response. This would be consistent with the observations that ROS production by Duox is vital for the effective elimination of ingested bacteria within *Drosophila* gut models; a mechanism which involved tight regulation to limit damage to host cells (Ha *et al.,* 2005a). This response is acknowledged to be extremely rapid, as expression of detoxification genes peak at one hour post infection with *Ecc15* (Buchon *et al.,* 2009). Lee *et al.* (2013) established that ROS production is induced in a Duox-dependent manner as early as one hour post infection with *Ecc15* in *Drosophila* gut models; taking into account the time taken for the bacterial stimulus to reach its gut target after the initial ingestion, this would again support the notion of a rapid ROS response to *Ecc15*. RT-qPCR results would suggest that expression of *Duox*, the enzyme responsible for epithelial ROS production, is downregulated in EcR^{Q50st} mutants, albeit at an insignificant level (data not shown). Considering the role of ROS in the rapid prosecution of bacteria, this result may suggest that *duox* expression lies downstream of 20-HE signaling through EcR-B1. Therefore, considering the necessity of a rapid ROS response upon *Ecc15* infection and the nature of the co-injection of *Ecc15* + 20-HE into Stage 11 embryos, one can speculate that this treatment does not permit sufficient time for Stage 11 embryos to initiate 20-HE signaling and manufacture functional Duox protein before the *Ecc15* infection reaches a threshold of growth which proves inevitably fatal to these early embryos. By contrast, this hypothesis would denote that Stage 15 embryos, having a ready supply of Duox potentially available as a result of the 20-HE pulse during mid-embryogenesis, would display greater resistance to *Ecc15* infection via the production of mucosal ROS. However, given the inconclusive nature of the RT-qPCR data regarding *duox* expression in

EcR^{Q50st} mutants upon *Ecc15* infection, further work would be required to test the biological significance of this concept.

In light of the importance of the trachea in mediating the AMP response to infection, as well as the requirement of functional EcR-B1 in the Stage 15 embryonic tracheal network to promote survival to infection and reduction of bacterial burden via activation, the hypothetical role of ROS in the eradication of *Ecc15* infection could be further supported. The trachea is armed with a diverse array of antioxidant enzymes (Wagner *et al.,* 2008). Genes related to the detoxification of ROS that are actively expressed in the airway epithelium include *Duox*, catalase, two superoxide dismutases, four peroxiredoxins and a range of glutathione S-transferases (Wagner *et al.,* 2008), alluding to the possibility that the airway epithelium produces a significant quantity of epithelial ROS that require subsequent detoxification. Thus, the production of ROS in the trachea could potentially be induced upon *Ecc15* infection and contribute to the host response to eliminate invading bacteria. On the other hand, the *Drosophila* trachea has been demonstrated to upregulate many other immune genes which could potentially lie downstream of 20-HE signaling and thus ease the burden of *Ecc15* infection. For instance, the *transferrin 1* gene has been demonstrated to be highly and specifically expressed within the trachea. Transferrin 1 is an iron-binding protein that is required to transport ionic iron (Yoshiga *et al.,* 1999), maintaining iron in soluble forms for its ultimate transport to cells for proliferation or biosynthesis of iron-requiring enzymes (Aisen, 1994). Due to its ability to bind and thus sequester iron, it has been postulated that transferrin may restrict bacterial access to iron, which is required for bacterial growth (Guerinot, 1994). This theory has been partially confirmed by the observation that *transferrin 1* expression is highly upregulated upon infection in *Drosophila* (Sackton and Clark, 2009); a phenomenon which has been observed in other insect infection models, such as the mosquito, *Aedes egypti* (Beerntsen *et al.,* 1994; Yoshiga *et al.,* 1997) and the termite *Mastotermes darwiniensis* (Thompson *et al.,* 2002). Its presence at a high concentration in the trachea suggests a vital role for iron depletion in the airway epithelium, which could potentially limit the growth of most bacterial species (Flo *et al.,* 2004). Moreover, the tracheal

network has been demonstrated to express ventral veinless (vvl), a transcription factor which has been shown to induce local AMP expression (Junell *et al.,* 2010), and thus may also play a role in the elimination of *Ecc15* infection specifically within the trachea. Further RT-qPCR studies to determine the transcriptional response of embryos deficient of these candidate genes upon infection would be required to elucidate whether activation of either of these candidate genes rely on 20-HE signaling within the *Drosophila* embryo.

5.2 The Role of psh in the Embryonic Immune Response

Whilst the majority of Stage 15 embryonic immune responses were demonstrated to be concordant with larval and adult fly models, there were a few notable exceptions. For instance, previous studies have shown that injection of *A. oryzae* proteases was unable to stimulate *Drs* expression in adult flies in a psh^{1} mutant background in comparison to a robust WT response (Buchon *et al.,* 2009b), which is presumably the reason why infection of psh^{1} mutant flies with *Aspergillus fumigatus* resulted in a more rapid rate of mortality than noted in WT or $modSP^{1}$ flies (Buchon *et al.,* 2009b). This effect on fly mortality was demonstrated to be exacerbated in $psh^{1};;modSP^{1}$ double mutant flies (Buchon *et al.* 2009b). Conversely, injection of *A. oryzae* proteases did not significantly reduce the viability of $psh^{1};;modSP^{1}$ Stage 15 embryos compared to that of WT controls; as such, $psh^{1};;modSP^{1}$ double mutants demonstrated greater survival levels to protease injection than single $modSP^{1}$ mutants alone, contrary to the results of Buchon *et al.* (2009b). *psh* is a host serine protease which is activated upon recognition of fungal and bacterial virulence factors, such as secreted proteases, and subsequently mediates Toll pathway activation and resistance to fungal infection (Ligoxygakis *et al.,* 2002; Gottar *et al.,* 2007; El Charmy *et al.,* 2008), and hence it would be expected that $psh^{1};;modSP^{1}$ mutant embryos should be more susceptible to treatment with such agents than WT or rel^{E20} mutant embryos. The fact that $modSP^{1}$ mutant embryos demonstrated a significant susceptibility to *A. oryzae* protease injection compared to WT embryos would suggest that this branch of the Toll pathway is functional within the Stage 15 embryo and able to mediate an appropriate resistant response to

fungal proteases. Thus the anomalous response noted in *psh*^{1}*;;modSP*^{1} embryos cannot be attributed to lack of *psh* functionality within the developing embryo. Therefore, mutation of *psh* seemingly improves the viability of *modSP*^{1} embryos post injection with A*. oryzae* proteases, and may infer that there is some biological benefit in lacking the ability to recognize fungal proteases within the *Drosophila* embryo that is distinct from the benefit incurred by modSP activation of the Toll pathway. Therefore, one way in which to test this hypothesis would be to conduct RT-qPCR experiments focusing on the transcription of AMP genes within Stage 15 WT, *modSP*^{1} and *psh*^{1}*;;modSP*^{1} embryos; to determine levels of AMP gene expression in the differential genetic backgrounds. Moreover, the dose of *A. oryzae* protease administered to embryos was low (1:5000) due to the high embryo mortality associated with injection of higher concentrations (data not shown), possibly leading to the large degree of variation observed between experimental replicates. Thus, repeating experiments using an increased dose of protease may further elucidate the degree of resistance of embryos to these virulence factors.

5.3. Infection and damage may exert long-term effects on development of embryonic systems and lifespan

It is clear from the data presented that the Stage 15 embryo transcriptome is regulated to adapt to the acute effects of infection and damage, and to meet the necessary demands that responses to these stimuli place upon the embryo. However, other elements of the data would suggest that these stimuli also have a much broader, long-term effect on the development of the embryos. For instance, infection with *Ecc15* or *M. luteus* induced the modulation of genes normally associated with development of the nervous system, such as *Rim, milt, snoo, bsh, caps, niki* and *Aats-thr*. This is further supported by the GO analysis, which demonstrated that genes upregulated by *Ecc15* were enriched for axon cargo transport and synaptic transmission functions and were potentially associated with a variety of neural components, such as the axon, synapse and presynaptic terminal. Similar associations

were noted between genes upregulated by *M. luteus* and GO terms relating to axon cargo transport function and an association with the synapse and membrane bound vesicles, which may be indicative of a role in neurotransmitter release. Moreover, the fact that these genes can be subdivided into those induced upon either infection with solely *Ecc15* (*Rim, milt, snoo*), only *M. luteus* (*bsh, Aats-thr*) or commonly by both types of infection (*niki, caps*) may suggest that distinct effects on embryonic nervous system development are mediated by individual infections, and that a combination of Gram-positive and Gram-negative bacteria also has a distinct effect. However, the precise mechanism by which this interaction between the immune and nervous systems occurs and the resulting effect this interaction has on the embryo is relatively speculative. Whilst the scientific literature has acknowledged a potential interaction between the immune and nervous systems, few studies have explored this potential interaction in depth. Work in vertebrates by Roitt *et al.* (1993) elucidated that several neuropeptides and biogenic amines produced in the central nervous system, such as Met-enkephalin, were also produced by immunocytes and had effects on lymphocyte proliferation and macrophage migration. Lavine and Strand (2002) postulated that the simultaneous arrangement of signaling and bacterial killing factors released via the processing of the opioid precursor protein could result in a rapid antibacterial response as well as a recruitment of immune cells and signaling of danger to the CNS. Lavine and Strand (2002) also speculate that a similar situation may exist in insects via ENF peptides which are processed in a similar manner to opioid precursor protein and are expressed in the CNS, fat body and immune cells, including hemocytes (Clark *et al.,* 1998; Hayakawa and Noguchi, 1998) This would be consistent with the observation that Stage 15 embryonic hemocytes also induce transcription of neurogenesis genes, such as *Rfx* and *Ndc80*, hence indicating a role for the hemocyte in the function or development of the nervous system post infection. Given the close spatial relationship between hemocytes and the ventral nerve cord within *Drosophila* embryos (Tepass *et al.,* 1994; Evans *et al.* 2010) and the hemocyte's reported role in the condensation of the central nervous system and development of the ventral nerve cord (Olofsson and Page, 2005; Evans *et al.,* 2010), it is interesting to speculate that hemocytes may be in a relevant

spatial position as well as functionally able to interact with the nervous system, merging its roles in nervous system development and cellular immunity. Thus, although an interaction between the nervous and immune systems has been documented, potentially corroborating results from our embryonic model, much more extensive work would be required to determine the precise contribution of hemocytes within this process and precisely what effect this relationship would have on resulting embryo fitness.

Another potentially interesting feature of this data relates to the upregulation of genes encoding odorant binding proteins (OBPs) and Odorant receptors (ORs) upon infection, specifically *M. luteus* infection. Members of the invertebrate OBP family are olfactory-specific molecules that are secreted from non-neuronal support cells into the lymph of subsets of olfactory sensilla (Galindo and Smith, 2001). OBPs are believed to play a role in the delivery of hydrophobic odorants through the lymph to the ORs (Carlson, 2001), although actual evidence of this concept remains scarce. *Drosophila* adults deficient in even one Obp gene display abnormal long-term olfactory behavioural responses to a variety of odorants (Kim *et al.,* 1998; Wang *et al.,* 2001), whereas overexpression of specific Or genes has been demonstrated to result in an elevation in the adult fly response to subsets of odours (Stortkuhl and Kettler, 2001). Thus, the upregulation of Obp family members by *M. luteus* infection within the embryo may represent a link between infection and subsequent behavioural responses of the resulting emergent larvae. This hypothesis could potentially be tested by assessing the olfactory response of larvae emerging post embryonic infection with *M. luteus* to different subsets of odours, and if overexpression or mutation of the Obp genes in question was able to alter this response. However, what potential advantage this purported change in behavior would confer upon subsequent larvae remains largely speculative; it is perhaps reflective of embryonic priming to potentially contaminated surroundings. Given that the natural *Drosophila* habitat for oviposition is dominated by decaying organic matter, which is likely to contain multiple and potentially harmful bacterial and fungal pathogens, the development of such a priming response would be highly beneficial to the developing embryo. This hypothesis is partially supported by the observation

that *C. elegans* modifies its olfactory preferences post infection with pathogenic bacteria to avoid odours from the pathogen and increase its attraction to odours from familiar non-pathogenic bacteria (Zhang *et al.,* 2005).

Moreover, the above results permit the speculation that both infection and damage may have more long-term effects, such as an effect on resulting adult lifespan. For instance, *Ecc15* and *M. luteus* infection in Stage 15 embryos were both demonstrated to upregulate the sorting nexin gene *snz*. Work by Suh *et al.* (2008) demonstrated that overexpression of *snz* in fat metabolic tissues reduced lifespan, whilst *snz* mutants exhibit prolonged youthfulness. Such results would suggest that infection has a negative effect on Stage 15 embryo lifespan, although the timescale over which this would potentially occur remains unknown. Results from Chapter 3 demonstrate that Stage 15 embryos are clearly affected by *Ecc15* infection in terms of viability post infection, although one could argue that this is due to the acute toxic effects of the *Ecc15* infection, as opposed to long-term effect on lifespan. However, considering the fact that fat-storing tissues, such as the fat body, can play a role in controlling lifespan (Picard and Guarente, 2005) as well as mediating a systemic immune response, it would perhaps not be surprising if there was some interplay between these two distinct processes. In fact, previous work has suggested that activated immunity appears to repress lifespan in *Drosophila* adults, whereas suppressed immunity appears to slow *Drosophila* aging (Paik *et al.,* 2012). This is a relationship that appears to also work in reverse, as studies have suggested that regimes such as dietary restriction which can increase lifespan (Bauer *et al.,* 2010) also impairs immune function and wound healing in mice (Reed *et al.,* 1996). Thus, the upregulation of *snz* by bacterial infection may simply further highlight the costliness of the immune response to the host.

However, it is also clear that the potential effect of infection on the resulting lifespan of the fly is more complex than a simple reduction as postulated above. For instance, increased levels of another gene upregulated upon *Ecc15* and *M. luteus* infection, *NIFR*, has previously been suggested to reduce reproductive behaviours and subsequently extend lifespan of adult flies (Paik

et al., 2012). Moreover, increased levels of IM3 expression, which were observed upon *M. luteus* infection alone within the Stage 15 embryos, has also been associated with an extension in lifespan (Bauer *et al.,* 2010). Thus, the increased levels of these candidate genes are relatively contradictory to the increase in expression of *snz* upon infection in the *Drosophila* embryo. It could be speculated that co-upregulation of genes with positive and negative effects on lifespan upon infection permits the host to maintain a balance, so that the deleterious effects of an immune response on lifespan are minimized.

The observation that sterile wounding also highly and significantly induces expression of *IM3* may also lead to the speculation that damage may have a positive effect on lifespan. However, this hypothesis is not consistent with the observation that increased damage via ROS is a critical causative factor in aging (Parkes *et al.,* 1998); considering the mechanical damage caused by ROS to cell components (Thannickal and Fanburg, 2000; Landolfo *et al.,* 2008) and DNA (Henmani and Parihar, 1998; Cooke *et al.,* 2003), as well as the observation that wounds release H_2O_2 (Niethammer *et al.,* 2009; Moreira *et al.,* 2010), it is therefore unlikely that the infliction of damage could have a positive effect of lifespan via the upregulation of *IM3*. As such, the observed upregulation of *IM3* expression by sterile laser ablation in Stage 15 embryos must fulfill another function. Previous evidence has suggested that *IM3* is regulated by the Toll pathway (De Gregorio *et al.,* 2001; Boutros *et al.,* 2002; Wertheim *et al.,* 2005), although the precise molecular function of this gene remains elusive. Therefore, these results may reinforce the notion that damage alone can induce activation of an immune response, potentially via the Toll pathway, as opposed to having any long-term effect on *Drosophila* lifespan. Patterson *et al*. (2013) demonstrated that puncture wounding of *Drosophila* embryos stimulated upregulation genes encoding Toll pathway components, including PGRP-SA, serine proteases such as spirit, AMPs such as Mtk and immune inducible molecules, such as IM1-4. This would potentially corroborate the above data, suggesting a role for Toll in mediating an immune response to damage. However, the nature of the puncture wound administered by Patterson *et al.* (2013) does not preclude for bacterial infection of the host during or after wounding, as the vitelline membrane of the

embryo is disrupted. Thus, it would be of interest to compare the transcriptional profile of embryos receiving PBS injection with those receiving sterile laser ablation treatment, to determine the extent of transcriptional overlap between these two different types of damage.

Therefore, as well as identifying candidate genes which may play a role in the acute response to infection or damage, the transcriptional studies performed in this project have also potentially identified genes which may impact on the long-term Stage 15 embryo development, behavior and lifespan. Further validation of these results, via RT-qPCR, will be required, as well as subsequent studies to assess the impact that these observed changes in gene expression may have on embryo functionality and fitness.

5.4 Final Conclusions

To conclude, the *Drosophila* embryo is a viable model system to study bacterial infection *in vivo*; Stage 15 embryos are able to mediate a relatively robust and complex response to different types of infection that is mostly concordant with defense mechanisms recorded in adult and larval models. Studies in the embryo have also permitted novel insight into the *Drosophila* immune response. For instance, whilst AMP production has thus far formed the cornerstone of *Drosophila* immunity studies and is doubtlessly important for a successful immune response, data from the embryo would suggest that it is not the only or most crucial factor in the elimination of bacterial pathogens. Furthermore, transcriptional profiling of infected and wounded embryos would suggest that the *Drosophila* immune response itself may have a long-lasting effect, even after the elimination of the pathogen; modulation of genes involved in lifespan, nervous system development and olfaction may suggest that infection and damage may have long-term effects on *Drosophila* development and behavior.

Appendix 1: Fly food and apple juice agar plate constituents

Table A1.1: Fly food constituents

Constituent	Amount per litre
Yeast extract	17g
Malt extract	39g
Maize flour	60g
Soya flour	9g
Plant agar	6g
Sugar	64g
Nippagin in 100% methanol	1.5g in 15mL
Propionic acid in 100% ethanol	5.3mL in 7.7mL

Table A1.2: Apple juice agar constituents

Constituent	Amount per litre
Plant agar	20g
Sucrose	12g
Apple juice	250mL
Nippagin in 100% ethanol	2g in 10mL

Appendix 2: Fly lines generated

Table A2.1: Fly lines generated for Chapter 3

Fly line	Use
w; srp-Gal4, UAS-GFP; crq-Gal4, UAS-GFP	RT-qPCR; mortality assay, CFU count assay
w; EcR^{Q50st}/CTG	Mortality assay
w; EcR^{M554fs}/CTG	Mortality assay
w; EcR^{Q50st}/EcR^{M554fs}	Mortality assay
w; EcR^{Q50st}/EcR^{Q50st}	Mortality assay; RT-qPCR
w; EcR^{M554fs}/EcR^{M554fs}	Mortality assay
w; UAS-EcR B1 DN/+; da-Gal4/+	Mortality assay
w; UAS-EcR B1 DN/srp-Gal4	Mortality assay
w; UAS EcR-B1 DN/btl-Gal4	Mortality assay; CFU count assay
w; UAS EcR-B1 DN/E22c-Gal4	Mortality assay
w; srp-Gal4, $scar^{\Delta 37}$/$scar^{\Delta 37}$; crq-Gal4, UAS-GFP	Phagocytosis assay, survival assay
w; srp-Gal4; crq-Gal, UAS-GFP	Phagocytosis assay, survival assay

Table A2.2: Fly lines used in Chapter 4

Fly line	Use
w; srp-Gal4, UAS-GFP; crq-Gal4, UAS-GFP	Generation of samples for microarray; preliminary hemocyte sorting studies
w; srp-Gal4; crq-Gal4	GFP-negative control for FACS

Appendix 3: Thermodynamic properties of Immune Gene Primers Generated

Table A3.1 Primers Generated using Primer3

Primer	Tm (°C)	GC content (%)	Pair any complementarity (AU)	Pair 3' complementarity (AU)
Ddc F	60.03	50.00	4.00	2.00
Ddc R	59.97	50.00	4.00	3.00
GADD45 F	60.37	61.11	4.00	0.00
GADD45 R	59.96	63.48	3.00	0.00
Mas F	56.00	43.00	4.00	0.00
Mas R	57.50	50.00	4.00	0.00

Appendix 4: DNA Folding Structures for Candidate Genes

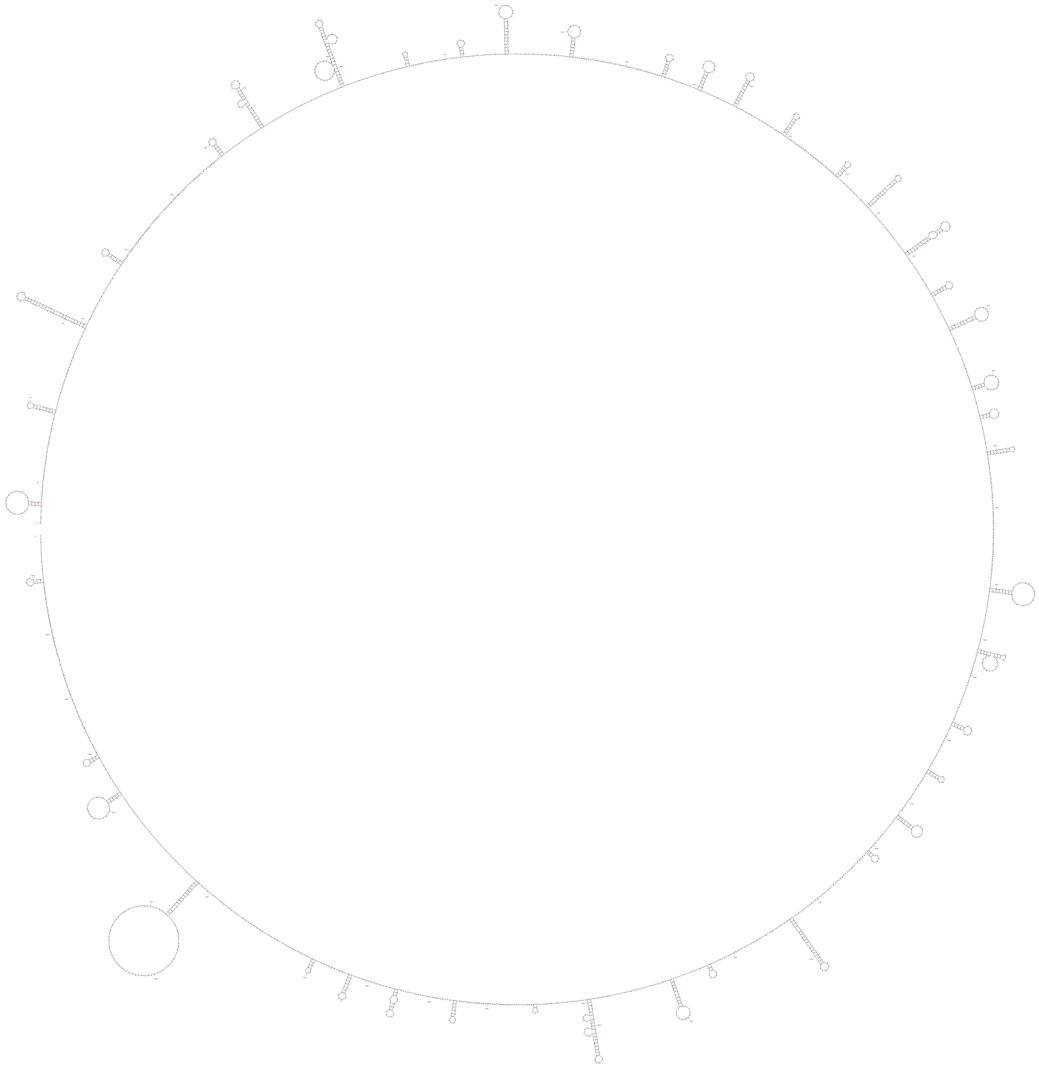


Figure A4.1 Mfold structure of *Ddc* at 60°C.

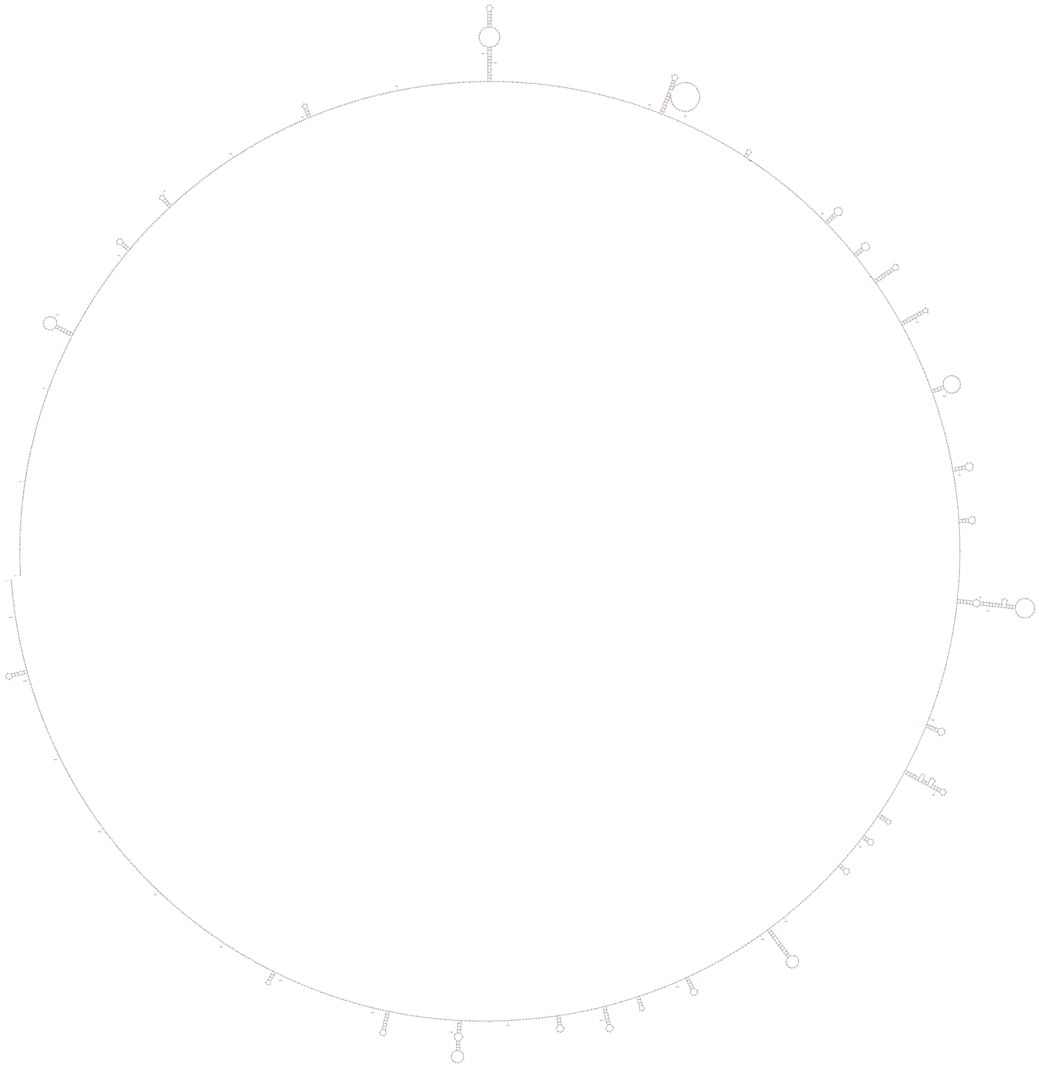


Figure A4.2 Mfold structure of *GADD45* at 60°C.

Figure A4.3 Mfold structure of *Mas* at 60°C.

Appendix 5: *In silico* assessment of primer specificity

Gene	Primer	Number of hits at high identity (AU)	Gene of interest selected? (Y/N)
Ddc	Forward	1	Y
	Reverse	1	Y
GADD45	Forward	1	Y
	Reverse	1	Y
Mas	Forward	1	Y
	Reverse	1	Y

Appendix 6: qPCR primer efficiencies

Table A6.1: Primer efficiency values of internal control primers

Primer pair	Mean efficiency (%)	Standard deviation (AU)
Rp49	89.09	2.65

Table A6.2: Primer efficiency values of Immune Primers Generated by Primer3

Primer pair	Mean efficiency (%)	Standard deviation (AU)
Ddc	103.73	19.73
GADD45	148.75	6.33
Mas	91.55	5.48

Table A6.3: Primer efficiency values of AMP primers

Primer pair	Mean efficiency (%)	Standard deviation (AU)
AttA	88.22	9.78
CecA1	83.22	12.34
Def	86.40	1.58
Dpt	95.78	12.18
Drc	91.04	6.73
Drs	101.91	12.78
Mtk	92.83	11.73

Table A6.4: Primer efficiency values for ecdysone signaling primers

Primer pair	Mean efficiency (%)	Standard deviation (AU)
Dare	85.01	1.77
Ecd	96.95	1.29
Eip71CD	81.79	0.86
Eip74EF	76.20	2.42
Eip78C	84.65	5.79
Eo	94.03	1.71
Mld	85.59	12.75
Nvd	88.38	0.40
Phm	96.68	7.72
Ptth	81.59	3.65
Sad	81.79	12.68
Usp	76.63	1.98

Appendix 7: Representative FACS Scatter Plots and Sort Data for the Collection of Hemocytes from Naïve, PBS and *Ecc15* treated embryos

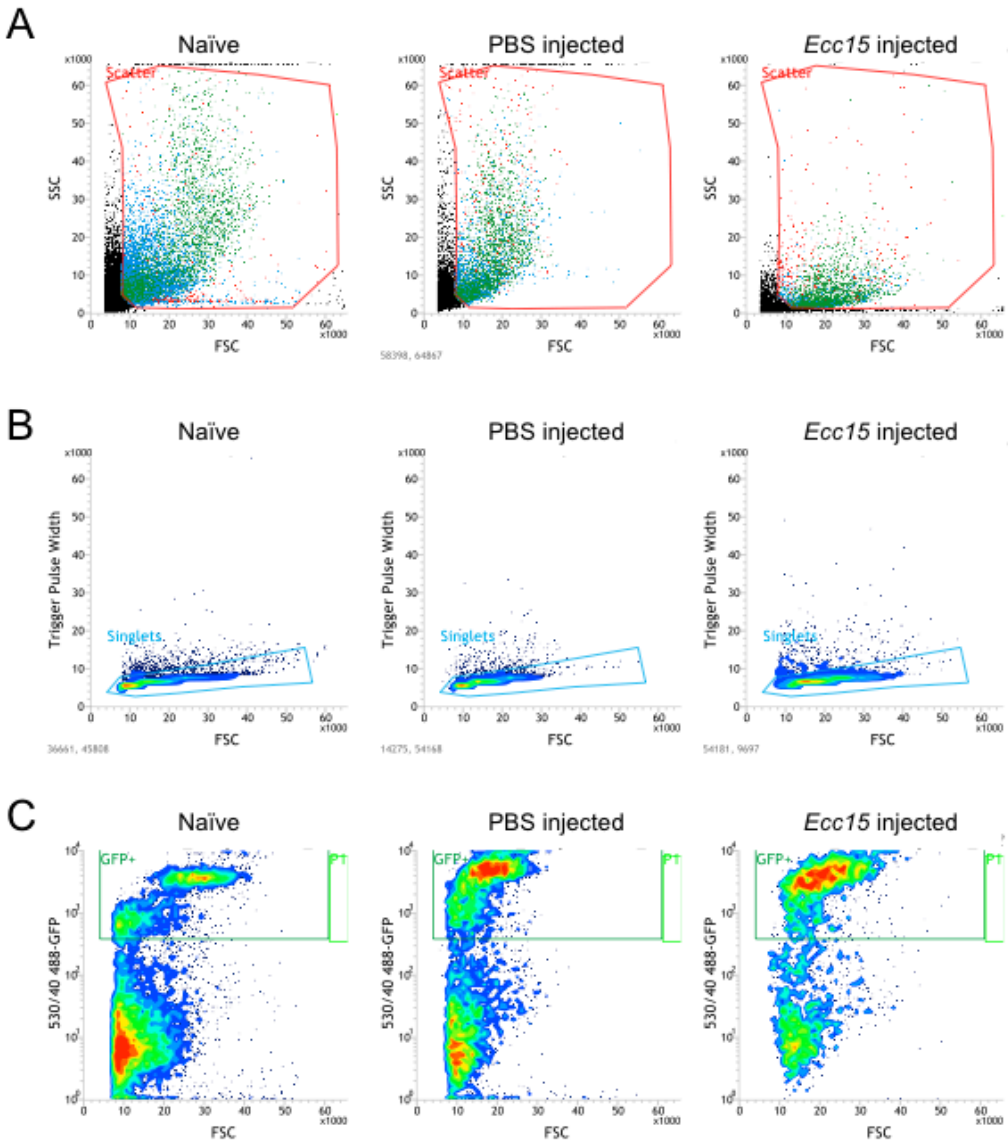


Figure A7.1: Representative Scatter Plots from FACS experiments to collect naïve and treated hemocytes for microarray studies. Gating for cells that demonstrated the correct size and complexity (A), singularity (B) and GFP-positive (GFP+) properties permitted the sorting of GFP-labelled hemocytes from naïve, PBS-injected and *Ecc15*-injected embryos.

Table A7.1: Hemocyte FACS Data quantifying the Percentage of Selected Cell Populations in the Total Cell Suspension

Sample	Total number of events (AU)	Percentage of Total Scatter (%)	Percentage of Singlets (%)	Percentage GFP+ cells (%)
Naïve 1	10000	39.17	35.13	9.74
Naïve 2	10000	38.01	32.56	8.24
Naïve 3	10000	35.39	33.5	9.26
Naïve 4	10000	33.84	30.96	6.07
PBS 1	10000	52.43	49.75	12.9
PBS 2	10000	45.16	42.09	19.59
PBS 3	10000	34.45	30.34	13.38
PBS 4	10000	40.8	36.47	7.78
Ecc15 1	10000	35.57	30.12	6.08
Ecc15 2	10000	35.18	30.95	7.75

Table A7.2: Numbers of Hemocytes Collected from Stage 15 Embryos during Different Treatments

Sample	Number of embryos (AU)	Total number of cells (AU)	Number of GFP+ cells (AU)
Naïve 1	1000	490842	43889
Naïve 2	1000	390000	23936
PBS 1	300	109670	13735
PBS 2	400	402000	31188
PBS 3	730	266830	16081
PBS 4	478	103772	13772
Ecc15 1	400	629000	36290
Ecc15 2	400	229746	26450

Appendix 8: Genes identified via transcriptional profile analysis that were uniquely upregulated in the Stage 15 embryo upon different types of bacterial infection

Table A8.1: *Ecc15*-unique upregulated genes within the Stage 15 embryo

Gene	Log Fold Change (AU)	Adjusted P-value
Adar	1.102	0.016
alpha-Man-I	1.641	0.011
AP-50	1.281	0.008
Aplip1	1.264	0.018
Art6	3.239	0.012
ash1	1.411	0.013
Atg9	1.468	0.016
AttA	4.936	0.010
AttC	3.592	0.005
AttD	3.867	0.006
beta'Cop	1.618	0.014
Bruce	1.338	0.016
CG10353	1.508	0.017
CG10505	2.495	0.013
CG10864	1.190	0.013
CG11263	2.214	0.004
CG11349	2.095	0.016
CG11529	2.372	0.005
CG11593	1.277	0.011
CG12162	1.446	0.010
CG12674	3.124	0.012
CG12692	2.274	0.013
CG13079	3.366	0.007
CG1407	1.089	0.017
CG14851	3.315	0.010
CG15450	4.867	0.018
CG15555	3.048	0.003
CG15831	1.932	0.015
CG15841 *CG6555*	2.538	0.007
CG16970	2.214	0.013
CG18171	1.467	0.017
CG2316	1.216	0.012
CG2336	2.553	0.008
CG31148	4.393	0.002
CG31178	2.274	0.003
CG31244	4.248	0.009
CG31538	2.060	0.011
CG31935	1.150	0.018

CG32079	3.488	0.018
CG32119	1.284	0.010
CG32148	2.988	0.003
CG32246	2.571	0.001
CG32679	3.441	0.017
CG32681	2.437	0.011
CG32732	1.146	0.016
CG32810	1.364	0.016
CG33156	1.968	0.011
CG33232	1.629	0.017
CG34349	1.686	0.010
CG3517	2.368	0.003
CG4165	1.565	0.013
CG42343	2.035	0.001
CG42389	1.594	0.013
CG42684	1.897	0.006
CG42750	2.687	0.001
CG43102	1.423	0.008
CG43689	1.497	0.005
CG4793	3.486	0.017
CG4996	1.087	0.013
CG5068	1.114	0.012
CG5720	1.334	0.012
CG7094	3.023	0.002
CG7180	1.660	0.015
CG7311	2.879	0.003
CG7337	1.184	0.011
CG7816	1.198	0.011
CG7927	1.165	0.013
CG8745	1.060	0.016
CG8918	6.404	0.001
CG9626	1.222	0.014
CG9692	2.603	0.004
Cip4	1.447	0.015
Cngl	1.699	0.012
CR10991	2.228	0.010
CR31386	2.075	0.015
Dref	1.469	0.008
Eag	4.121	0.005
Edin	3.753	0.018
Fatp	1.077	0.018
Fili	1.705	0.008
fl(2)d	1.522	0.013
Fne	1.342	0.016
gcm2	1.849	0.016
Glo	2.229	0.013
HP5	1.364	0.007
HPS4	1.347	0.010

Hsf	1.174	0.014
l(3)02640	1.518	0.015
MAPk-Ak2	1.064	0.017
Mgat1	1.454	0.012
Milt	1.252	0.013
Mst77F	1.859	0.015
Mun	3.113	0.006
Na	2.257	0.014
Orc1	1.678	0.013
Patr-1	1.091	0.016
PGRP-LF	1.112	0.013
Pkd2	3.588	0.009
PRL-1	1.038	0.016
proPO-A1	1.078	0.014
pyd	1.388	0.018
r-cup	1.922	0.002
Rab9	3.872	0.011
RhoGAP1A	1.405	0.016
Rim	2.406	0.007
Sema-2a	1.439	0.005
SIP3	2.794	0.011
Smg6	1.151	0.014
Snoo	1.295	0.008
spaw	1.747	0.009
TBPH	1.456	0.016
tkv	1.298	0.015
Trf4-2	1.965	0.014
vap	1.176	0.016
Vha100-3	2.169	0.012
Vps26	1.507	0.008
wb	1.565	0.014

Names highlighted in green indicate a gene of unknown function.

Table A8.2: *M. luteus*-unique upregulated genes in the Stage 15 embryo

Gene	Log Fold Change (AU)	Adjusted P-value
2mit	1.310	0.009
Aats-thr	1.211	0.018
Acp53Ea	1.954	0.012
Amph	1.011	0.022
aralar1	1.277	0.022
Arr2	1.448	0.009
Atf-2	2.281	0.012
bsh	1.874	0.014
CG10185	1.650	0.008
CG10211	1.025	0.018
CG10494	1.201	0.017
CG10663	1.343	0.018
CG10801	1.635	0.012
CG10814	4.500	0.006
CG10947	1.042	0.019
CG11162	4.446	0.003
CG11665	1.006	0.021
CG12278	1.218	0.010
CG12413	1.334	0.013
CG12703	1.218	0.018
CG13248	1.252	0.015
CG13300	1.386	0.014
CG13325	6.971	0.003
CG13359	2.402	0.008
CG13575	2.904	0.018
CG13611	2.622	0.013
CG13796	1.847	0.016
CG13796	2.440	0.009
CG14183	2.047	0.018
CG14219	3.178	0.005
CG14294	2.658	0.005
CG14383	4.227	0.013
CG14565	2.407	0.016
CG14691	2.034	0.002
CG14778	1.320	0.013
CG14803	1.559	0.020
CG15332	3.190	0.019
CG15625	3.245	0.010
CG15630	1.273	0.011
CG15824	4.425	0.014
CG16800	1.726	0.019
CG1774	1.356	0.009
CG18810	4.527	0.011
CG1950	3.349	0.004
CG2129	2.891	0.020
CG2574	2.819	0.004

CG30127	2.995	0.002
CG31050	2.130	0.013
CG31053	3.123	0.018
CG3108	1.817	0.011
CG31089	2.970	0.002
CG31413	3.008	0.008
CG31475	1.290	0.011
CG31698	3.997	0.006
CG31752	1.954	0.012
CG32295	2.188	0.006
CG32668	1.339	0.016
CG32709	1.853	0.004
CG33235	3.381	0.001
CG33262	2.990	0.016
CG33286	3.904	0.005
CG34113	1.977	0.015
CG34127	1.942	0.020
CG42594	4.222	0.005
CG42810	2.386	0.008
CG43347	1.816	0.005
CG43693	1.038	0.018
CG5791	2.074	0.021
CG5877	1.172	0.021
CG5987	1.872	0.007
CG6123	1.268	0.021
CG6133	1.508	0.016
CG6499	1.811	0.010
CG6652	2.230	0.016
CG6839	3.800	0.009
CG7166	1.144	0.015
CG8064	1.348	0.016
CG8172	1.414	0.019
CG8500	1.143	0.019
CG8642	3.888	0.015
CG8784	2.128	0.007
CG8909	1.207	0.013
CG9576	1.965	0.019
CG9602	1.731	0.002
Clk	2.802	0.017
Cyp308a1	3.185	0.008
Cyp309a2	1.528	0.008
dpr16	2.595	0.021
Eig71Eh	3.380	0.002
fusl	1.602	0.017
gce	2.146	0.016
Gld	1.442	0.013
Hil	1.457	0.010
mam	2.737	0.018

Mctp	1.552	0.013
Mst36Fa	2.280	0.010
nito	2.670	0.022
Obp57e	2.833	0.006
Or85d	5.442	0.003
Or98a	2.457	0.005
Pif1A	2.824	0.005
ple	1.195	0.019
Porin2	2.253	0.006
PsGEF	2.126	0.002
RpL8	1.807	0.015
sut3	5.509	0.015
Synd	1.145	0.015
Sytbeta	1.448	0.008
Tepl	5.084	0.007
Tim17a1	2.696	0.001
Xpd	1.137	0.021

Names highlighted in green indicate a gene of unknown function.

Appendix 9: The 100 Most Significantly Modulated Genes upon Sterile Laser Ablation of the Stage 15 Embryo

Table A9.1

Gene name	Log Fold Change (AU)	Adjusted P-value
CG13044	3.28	3.65E-07
CG32548	3.44	1.23E-06
CG42305 *CG17325*	2.75	1.42E-06
5SrRNA-Psi:CR33363	2.44	2.58E-06
TwdlY	3.25	2.62E-06
snRNA:U5:38ABb *snRNA:U5:63BC* *snRNA:U5:14B* *snRNA:U5:38ABa* *snRNA:U5:35D* *snRNA:U5:23D* *snRNA:U5:34A*	2.38	3.26E-06
CG10827	2.35	3.27E-06
Lcp65Ad	3.62	3.35E-06
Obp56d	2.31	3.36E-06
CG15650	3.08	3.54E-06
CG9269	2.24	3.90E-06
CG15731	2.59	4.22E-06
CG9757	2.75	4.53E-06
CG14075	2.27	4.68E-06
CG7548	3.05	4.87E-06
CG2816	2.30	4.97E-06
CG15615	3.13	5.05E-06
Cpr65Av	2.83	5.55E-06
obst-E	2.33	5.57E-06
Cpr62Bc	3.04	7.12E-06
CG13043	2.15	7.28E-06
CG8629	3.26	7.58E-06
CG13038	2.06	7.91E-06
TwdlV	2.74	8.03E-06
TwdlK	3.67	8.44E-06
CG17127	2.81	8.78E-06
CG5070	3.21	9.27E-06
TwdlM	2.68	1.01E-05
CG12546 *CG14452*	4.01	1.02E-05
y	2.11	1.03E-05
CG7402	2.58	1.03E-05
CG42808	2.03	1.07E-05
CG7080	1.97	1.17E-05

CG32453	2.97	1.27E-05
CG13041	2.08	1.30E-05
snRNA:U5:38ABb *snRNA:U5:63BC* *snRNA:U5:14B* *snRNA:U5:38ABa* *snRNA:U5:35D* *snRNA:U5:23D* *snRNA:U5:34A*	2.34	1.39E-05
CG16885	1.93	1.41E-05
CG15127	1.84	1.42E-05
Obp56h	2.03	1.44E-05
Cralbp *Pmi* *PGRP-LD*	2.15	1.51E-05
CG13218	2.03	1.52E-05
CG14752	3.02	1.53E-05
Cpr50Cb	1.88	1.59E-05
levy	1.95	1.62E-05
CG8563	1.84	1.65E-05
CG14147	3.15	1.76E-05
CG7330	1.94	1.76E-05
CG15649	2.76	1.81E-05
snRNA:U5:38ABb *snRNA:U5:63BC* *snRNA:U5:14B* *snRNA:U5:38ABa* *snRNA:U5:35D* *snRNA:U5:23D* *snRNA:U5:34A*	2.34	1.89E-05
CG10657	1.85	1.90E-05
CG12655	2.21	1.94E-05
yellow-e	1.92	1.97E-05
CG9083	3.05	1.99E-05
CG12310	2.89	2.06E-05
snRNA:U5:38ABb *snRNA:U5:63BC* *snRNA:U5:14B* *snRNA:U5:38ABa* *snRNA:U5:35D* *snRNA:U5:23D* *snRNA:U5:34A*	2.23	2.18E-05
CG6870	2.16	2.24E-05
Ccp84Ad	3.05	2.28E-05
CG13058	1.72	2.34E-05
obst-E	2.57	2.35E-05
Cpr62Bb	3.16	2.42E-05
CG43386	2.04	2.45E-05
CG31198	1.74	2.69E-05

CG14770	2.00	2.70E-05
RpLP2	2.15	2.80E-05
CG15829	1.88	2.82E-05
Cpr78Cc	2.64	2.88E-05
CG13102	2.69	2.92E-05
Muc30E	-1.85	2.94E-05
Phk-3	1.98	2.99E-05
Cpr51A	1.85	3.01E-05
Cpr56F	3.66	3.05E-05
TwdlQ	4.02	3.05E-05
TwdlB	2.12	3.21E-05
CG2157	2.27	3.39E-05
CG13297	1.72	3.67E-05
Lcp65Ae	2.17	3.72E-05
CG13285	1.94	3.86E-05
CG9686	1.96	3.88E-05
CG4998	1.97	3.95E-05
CG2162	1.65	4.02E-05
snRNA:U1:82Eb	1.91	4.13E-05
Odc1	2.31	4.14E-05
IM3	1.74	4.18E-05
TwdlX	1.76	4.18E-05
b6	2.24	4.20E-05
Lcp65Ag3	2.28	4.44E-05
CG13331	2.13	4.47E-05
CG13049	3.36	4.49E-05
CG7031	1.74	4.50E-05
CG30101	1.78	4.59E-05
TwdlBeta	4.54	4.60E-05
Vago	1.59	4.66E-05
Osi10	1.60	4.88E-05
CG5506	1.86	4.96E-05
CG2901	-1.89	5.04E-05
Obp47b	1.65	5.04E-05
CG13047	2.60	5.25E-05
TwdlG	1.65	5.31E-05
CG6055	2.24	5.37E-05
CG8888	1.61	5.48E-05

Names highlighted in green indicate genes of unknown function. Names highlighted in red indicate downregulated genes.

Appendix 10: 100 Most Modulated Genes upon damage inflicted via PBS injection

Table A10.1: 100 Most Upregulated Genes upon PBS injection

Gene name	Log fold change (AU)
Acp65Aa	2.640
Ccp84Ad	2.961
Ccp84Ae	3.276
CG10472	2.369
CG10657	2.330
CG10911	2.279
CG11147	2.337
CG11159	2.335
CG11911	2.735
CG12546 *CG14452*	2.946
CG13026	2.651
CG13041	3.146
CG13042	3.366
CG13043	2.881
CG13044	4.010
CG13048	2.959
CG13049	2.230
CG13059	2.930
CG13060	2.612
CG13063	3.633
CG13066	4.480
CG13067	3.402
CG13068	3.437
CG13069	3.760
CG13217	2.563
CG13218	3.059
CG13239	3.228
CG13314	2.290
CG13463	2.621
CG13618	2.425
CG13674	3.786
CG13678	3.941
CG13679	3.106
CG13731	2.922
CG14095	5.161
CG14096	2.410
CG14147	3.497
CG14205	2.599

CG14327	2.911
CG14369	2.213
CG14752	3.106
CG15212	3.436
CG15213	3.717
CG15249	3.346
CG15615	2.978
CG15649	2.540
CG15650	2.622
CG16820	2.743
CG16886	2.700
CG17290	2.722
CG18294 *CG12519* *CG14096*	2.412
CG18649	2.288
CG30101	2.376
CG32266	2.949
CG32453	2.316
CG32829	2.221
CG43386	2.574
CG4962	3.364
CG5070	2.765
CG5326	2.230
CG6277	4.355
CG7402	2.896
CG7548	2.930
CG8541	2.492
CG9192	2.857
CG9686	2.425
Cpr100A	2.332
Cpr62Bc	3.444
Cpr64Ab	3.582
Cpr65Av	3.039
Cpr65Ax1 *Cpr65Ax2*	3.919
Cpr65Ea	3.307
Cpr65Ec	2.220
Cpr67Fa1	2.411
Cpr78Cc	2.999
drd	2.253
Lcp65Ac	2.372
Lcp65Ad	2.466
Lcp65Ae	3.262
Lcp65Af	2.890
Lcp65Ag1	3.488
Lcp65Ag2	3.867
Lcp65Ag3	3.325

Obp47b *CG7741*	2.246
Obp56d	2.439
obst-E	3.219
Tb	2.232
Tsp42Er	2.502
TwdlB	3.679
TwdlBeta	3.699
TwdlC	2.390
TwdlD	3.083
TwdlK	3.249
TwdlL	4.007
TwdlM	3.054
TwdlN	2.621
TwdlP	2.319
TwdlY	2.464
y	2.500
yip	2.610

Table A10.2: 100 Most Downregulated Genes upon PBS injection

Gene name	Log fold change (AU)
Abl	-1.190
Art4	-1.315
bol	-1.422
CG10005	-1.268
CG10107	-1.278
CG10348	-1.196
CG11029	-1.358
CG11966	-1.228
CG12009	-1.205
CG12050	-1.284
CG12288	-1.319
CG1273	-1.566
CG13096	-1.164
CG13361	-2.540
CG13362	-2.647
CG13622	-1.443
CG13983	-1.850
CG14418	-1.937
CG14803	-1.220
CG15927	-1.334
CG16798	-1.165
CG17549	-1.176
CG17672	-1.404
CG17780 *CG17781*	-1.223
CG17782	-2.687
CG17784	-2.072
CG18810	-1.246
CG3108	-1.221
CG31244	-1.177
CG31321	-1.453
CG32642	-1.379
CG32643	-1.259
CG32793	-1.613
CG34350	-1.197
CG34428	-2.229
CG4116	-3.288
CG4164	-1.172
CG42542	-1.208
CG43164	-2.219
CG43366	-1.253
CG4558	-1.160
CG4702	-1.255
CG5150	-1.302

CG5391	-1.381
CG5656	-1.939
CG5762	-1.321
CG5776	-1.503
CG6034	-1.566
CG6118	-1.781
CG6133	-1.523
CG7173	-1.464
CG7300	-1.631
CG7744	-1.215
CG8420	-1.761
CG8483	-1.760
CG8878	-1.209
CG9143	-1.296
CG9592	-1.227
CG9993	-1.356
Cht5	-1.364
Cpr47Ef	-1.211
Cpr65Az	-1.556
CR10102 *Arc1*	-1.183
Csat	-1.376
Cyp4ad1	-1.223
dy	-1.212
Eip71CD	-1.568
fbl6	-1.185
GalNAc-T2	-1.164
glo	-1.355
hiw	-1.212
Hsp26	-1.264
Hsp67Bb *Hsp22*	-1.535
Hsp68	-1.335
Hsp70Aa *Hsp70Ab*	-1.697
Hsp70Ba *Hsp70Bb* *Hsp70Bbb* *Hsp70Bc,*	-2.413
Hsp70Bb *Hsp70Bbb* *Hsp70Bc*	-1.925
ImpE2	-1.323
jhamt	-1.234
lqfR	-1.205
Lsp2	-1.445
mas	-1.333
mei-217 *mei-218*	-1.222

neo	-1.636
NijA	-2.070
NT1	-1.225
nyo	-1.220
Orc1	-1.267
Osi3	-1.173
Patj	-1.205
pip	-1.575
Sas10	-1.240
sha	-1.353
shd	-1.243
Snm1	-1.202
spag4	-1.261
Tango13	-1.722
Wnt2	-1.160
Wnt 4	-1.579

Appendix 11: 100 Most Modulated Genes within Hemocytes upon *Ecc15* Infection

Table A11.1: 100 Most Upregulated Genes within Hemoocytes upon *Ecc15* Infection

Gene name	Log Fold Change (AU)
Ir41a,	11.454
Ugt86De,	11.153
CG31898,	10.924
CG18213,	10.474
Pde1c,	10.112
CG42284,	9.799
Rab9Db,	9.782
AGO3,	9.744
pcl,	9.536
CG10344,	9.469
CG15756,	9.424
CG7432,	9.346
CG7365,	9.319
CG15894,	9.101
CG15044,	8.932
Ccap,	8.930
CG15531,	8.877
CG9903,	8.536
OS9,	8.533
ninaG,	8.407
Nxf3,	8.360
CG42237,	8.265
CG13073,	8.187
CG3884,	8.049
Dh44-R2,	8.026
Cht6,	7.953
dnd,	7.923
CG13562,	7.883
CG30196,	7.866
GstD6,	7.858
CG11362,	7.836
CG33093,	7.804
CG3823,	7.737
CG13813,	7.691
CG16898,	7.663
fd96Ca,	7.579
CG4267,	7.561
Cyp4p1,	7.553
phm,	7.552
CG13278,	7.537

Tsp42Er,	7.461
CG17118,	7.441
pad,	7.329
Rfx,	7.321
CG16998,	7.305
sisA,	7.130
Ac3,	7.104
CG42564,	7.099
CG13068,	7.088
CG14965,	7.055
CG4377,	6.993
CG17374,	6.986
Ndc80,	6.959
Oatp33Eb,	6.954
CG13036,	6.928
CG14036,	6.910
CG42797,	6.843
CG14451,	6.839
CG43143,	6.808
Best1,	6.745
CG11191,	6.729
ebd2,	6.715
Cht6,	6.587
CG13035,	6.554
RhoGAP18B,	6.486
CG31370,	6.338
CG3085,	6.303
tld,	6.238
CG9406,	6.216
CG31832,	6.211
Nep5,	6.162
Corin,	6.095
CG1090,	6.077
Or67d,	5.993
CG10555,	5.970
CG18371,	5.968
CG43337,	5.936
CG30016,	5.881
CG3556,	5.816
Antp,	5.784
Acox57D-d,	5.744
bnl,	5.716
mthl9,	5.678
CG13889,	5.553
CG12912,	5.492
b6,	5.469
CG14314,	5.466
CG33514,	5.447

mei-W68,	5.422
CG9427,	5.375
CG11475,	5.374
CG11899,	5.355
CG11160,	5.347
mspo,	5.336
CG31636,	5.310
CG17065,	5.184
CG4301,	5.057
boi,	5.048
Gr39a,	5.042
Cyp4d8,	5.016

Table A11.2: 100 Most Downregulated Genes in Hemocytes upon *Ecc15* injection

Gene name	Log Fold Change (AU)
CR31032,	-6.619
Brca2,	-6.433
CG11619,	-6.113
CG33178,	-6.053
CG4860,	-5.944
CG11504,	-5.859
alpha-Est10,	-5.807
CG17047,	-5.655
fz4,	-5.596
dgt5,	-5.580
CG42331,	-5.573
zfh2,	-5.475
CG14814,	-5.391
CG8924,	-5.326
Rnp4F,	-5.304
CG6912,	-5.271
CG7441,	-5.252
CG8944,	-5.230
RhoGAP102A,	-5.185
ckd,	-5.155
CG43462,	-5.142
Dup99B,	-5.033
qless,	-5.026
CG7497,	-4.987
Sry-alpha,	-4.972
CG34388,	-4.962
CG10654,	-4.956
sec15,	-4.946
CG2258,	-4.944
wb,	-4.940
CG4741,	-4.936
CG42255,	-4.908
spn-B,	-4.902
fd59A,	-4.897
CG34133,	-4.895
CG40198,	-4.880
Cap-H2,	-4.876
Mal-A8,	-4.855
CG31414,	-4.851
CG34449,	-4.840
Cks30A,	-4.839
Dl,	-4.814
CG30460,	-4.777
vn,	-4.776

cpo, *CG42457,*	-4.775
Rad9,	-4.744
CG15005,	-4.744
insc,	-4.734
CG15120,	-4.720
hts,	-4.708
Sh,	-4.704
CG42588,	-4.673
CG4607,	-4.663
CG8964,	-4.662
bru-2,	-4.659
CG5205,	-4.653
CG30001,	-4.634
CG3502,	-4.633
CG4269,	-4.627
CG13847,	-4.625
ImpE3,	-4.622
CG33203,	-4.619
CG4570,	-4.596
Zasp66,	-4.586
CG11029,	-4.582
ss,	-4.573
CG31871,	-4.571
slow,	-4.566
CG13609,	-4.562
CG31812,	-4.559
CG10200,	-4.551
cindr,	-4.536
Argk,	-4.528
CG33107,	-4.513
HLH4C,	-4.511
CG16972,	-4.492
CG4020,	-4.486
ZAP3,	-4.485
CG32191,	-4.477
CG30026,	-4.474
CG18476,	-4.461
CG16868,	-4.460
CG12728,	-4.410
CG13506,	-4.408
CG4942,	-4.403
rt,	-4.386
exex,	-4.371
CG40498,	-4.368
Pif1B, *Pif1A,*	-4.367
GRHR,	-4.367

phl,	-4.359
Rpt3R,	-4.349
CG11755,	-4.334
CG8519,	-4.324
CG7102,	-4.319
Spn43Ab,	-4.311
neb,	-4.290
CG3117,	-4.289
Hr39,	-4.288
CG8632,	-4.284

Appendix 12: 100 Most Modulated Genes within Hemocytes upon PBS Injection

Table A12.1: 100 Most Upregulated Genes in Hemocytes upon PBS injection

Gene name	Log Fold Change (AU)
Pde1c,	12.237
CCHa2r,	11.983
CG33093,	11.941
CG17270,	11.267
Tsp42Er,	11.217
Cpr97Ea,	10.691
CG30272,	10.679
fan,	10.656
Pka-C3,	10.465
CG30279,	10.444
CG14142,	10.431
CG6723,	10.431
Gr85a,	10.372
nub,	10.318
CG40485,	10.256
CG10344,	10.185
Cpr66Cb,	10.164
zwilch,	9.877
CG11966,	9.875
Cpr64Aa,	9.850
Cyp6t1,	9.831
CG42686,	9.733
CG31370,	9.584
CG40485,	9.555
PGRP-LB,	9.134
CG5107,	9.028
CG31102,	8.910
CG7142,	8.899
Ugt86Da,	8.853
CG14872,	8.742
CG9203,	8.610
CG43347,	8.569
laccase2,	8.290
Or1a,	8.217
Best1,	8.192
CG15021,	7.991
Gyc-89Db,	7.954
CG2990,	7.938
CG43078,	7.934
CG32368,	7.921
Corin,	7.856

CG10362,	7.802
CG15829,	7.801
CG42797,	7.790
CG16700,	7.772
Buffy,	7.646
CG6129,	7.639
lz,	7.569
mspo,	7.551
nvd,	7.548
CG13101,	7.542
CG14245,	7.483
Cyp4p1,	7.459
Nrk,	7.452
CG6208,	7.346
CG6175,	7.286
rho-5,	7.185
CG43337,	7.159
CR17025,	7.066
Tie,	7.062
CG9194,	7.050
ChLD3,	7.021
CG11505,	7.011
CG3987,	6.993
CG7991,	6.971
CG15423,	6.971
rdo,	6.916
TpnC41C,	6.902
CG14223,	6.875
Ggamma1,	6.799
CG4377,	6.794
Ag5r,	6.752
cpx,	6.728
CG17378,	6.607
CG6347,	6.462
CG14441,	6.444
CG40467,	6.416
mnb,	6.357
Sgs8,	6.324
CG31064,	6.284
CG4991,	6.260
Pxd,	6.215
CG6041,	6.197
X11Lbeta,	6.079
CG30002,	6.033
CG11120,	6.004
Sh,	5.997
Tsp42Ec,	5.972
phm,	5.949

sog,	5.927
mbl,	5.892
Vrp1,	5.844
dia,	5.821
Cyp316a1,	5.818
CG15393,	5.752
CG15930,	5.694
CG14079,	5.630
tou,	5.607

Table A12.2: 100 Most Downregulated Genes in Hemocytes upon PBS injection

Gene name	Log Fold Change (AU)
Pk92B,	-4.895
scro,	-4.809
Faa,	-4.717
CG7328,	-4.686
CG4393,	-4.655
CG11164,	-4.591
CG31414,	-4.570
CG2292,	-4.560
CG7441,	-4.543
CG13810,	-4.515
Rlb1,	-4.509
CG14980,	-4.503
CG9992,	-4.462
Scgbeta,	-4.446
Rad17,	-4.434
CG6891,	-4.422
Fs(2)Ket	-4.405
CG8300,	-4.399
Liprin-gamma,	-4.379
Tbp,	-4.371
Tsp39D,	-4.371
nej,	-4.361
Eaf,	-4.354
CG9471,	-4.342
CG15535,	-4.340
Snp,	-4.282
Ppox,	-4.281
CG13933,	-4.277
Unc-115a,	-4.271
scrt,	-4.258
CG7442,	-4.252
tef,	-4.226
CG7759,	-4.214
Top2,	-4.185
synj,	-4.178
CG16868,	-4.175
CG10107,	-4.161
Upf2,	-4.128
pug,	-4.098
Or71a,	-4.094
Aats-ile,	-4.088
Ncc69,	-4.086
CG12155,	-4.051
CG32040,	-4.039

CG10005,	-4.019
CG8569,	-4.017
CG43340,	-4.001
net,	-3.997
cnc,	-3.995
RhoGEF4,	-3.990
CG32104,	-3.990
CG9114,	-3.981
Dip3,	-3.974
CkIIbeta,	-3.962
CG11986,	-3.958
RpI135,	-3.951
CG14619,	-3.944
CG5618,	-3.932
Trf,	-3.928
stv,	-3.921
CG42588,	-3.908
Zasp52,	-3.904
CG9305,	-3.866
CG13188,	-3.856
CG9630,	-3.848
CASK,	-3.846
CG7497,	-3.841
Trc8,	-3.841
Fbw5,	-3.836
CG17360,	-3.832
Taspase1,	-3.812
CG4741,	-3.801
Itp-r83A,	-3.798
Xpd,	-3.796
CG6398,	-3.795
CG8927,	-3.791
CG1998,	-3.789
Vps13,	-3.785
Try29F,	-3.784
CG13163,	-3.758
l(1)G0469,	-3.751
r-cup,	-3.744
CG4730,	-3.740
f-cup,	-3.739
CG11983,	-3.729
CG30460,	-3.727
Hydr2,	-3.723
pex1,	-3.715
CG3194,	-3.711
Cyp28a5,	-3.694
CG16863,	-3.680
aux,	-3.674

Blimp-1,	-3.667
CG3880,	-3.662
Hsf,	-3.659
Glut1,	-3.659
CG31871,	-3.648
CG3407,	-3.642
Lim1,	-3.640
lbl,	-3.638

Appendix 13: Volcano Plots Demonstrating Significant Changes in Global Gene Expression upon Bacterial Infection and Damage

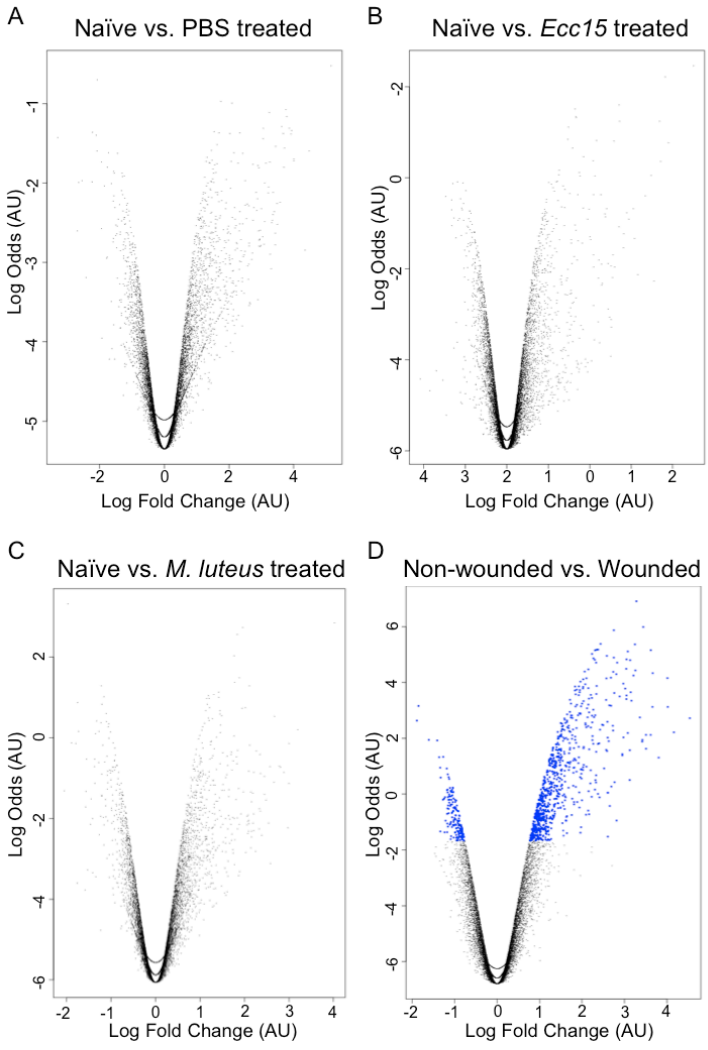


Figure A12.1: Volcano plots to show significant changes in global gene expression upon bacterial infection and damage. After vsn normalization of the microarray data sets, the significance of changes in expression between Naïve and injected (A-B) or wounded and non-wounded (D) was calculated. Blue dots indicate genes whose expression was significantly modulated upon treatment compared to Naïve/Unwounded levels ($p \leq 0.05$). Significant changes in gene expression were only noted when comparing non-wounded and wounded embryo transcriptional profiles (D), whereas all other treatments did not result in significant changes in expression (A-C).

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